AN ABSTRACT OF THE THESIS OF

Leena Leelasvatanakij for the degree of Master of Science in Pharmacy presented on August 6, 1993.

Title: Comparison and Optimization of Chromatographic Conditions for Separation of Cyclic Dynorphin A Analogues from Linear Byproducts.

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Abstract approved: _____
Jane V. Aldrich

The preparation of conformationally constrained peptide analogues can be complicated by difficult separations of the desired cyclic peptide from byproducts produced during the cyclization reaction. In this study, chromatographic conditions for the separation of the lactam analogue of dynorphin (Dyn) A (1-13)NH_2 cyclo[D-Asp^2,Orn^5]Dyn A (1-13)NH_2 from the tetramethylguanidinium (Tmg) byproduct [D-Asp^2,Orn(Tmg)^5] Dyn A (1-13)NH_2 and linear peptide [D-Asp^2,Orn^5]Dyn A (1-13)NH_2 were compared. The Tmg byproduct was obtained following the cyclization of the peptides with 2-(1-H-benzotriazo-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU). Two methods of high performance liquid chromatography (HPLC), reverse phase (RPC) and ion-exchange (IEC) chromatography, were examined. A number of factors affect the resolution and the elution profile of peptides, including the ion-pairing

agent, the mobile phase pH and the organic solvent. These factors were varied to obtain optimum separation. The separation of the Tmg side product, obtained from the cyclization with HBTU, from the cyclic peptide was more difficult than the separation of the linear peptide [D-Asp^2,Orn^5]Dyn A(1-13)NH_2 from the cyclic peptide. The order of elution of the peptides differed on RPC versus IEC; the cyclic peptide was eluted after the Tmg and linear byproducts in RPC while the order was reversed in IEC. In RPC, the standard conditions of 0.1% TFA in aqueous AcCN were not optimum and the separation was enhanced by using triethylammonium phosphate (TEAP) buffer pH 2.5 in aqueous AcCN, 0.1% H_3PO_4 in aqueous AcCN or 0.1% TFA in aqueous MeOH. In IEC, the best separation was achieved with a KCl gradient (100-600 mM) in phosphate buffer pH 3.0 containing 10% AcCN.

Comparison and Optimization of Chromatographic Conditions for Separation of Cyclic Dynorphin A Analogues from Linear Byproducts

by

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COMPARISON AND OPTIMIZATION OF CHROMATOGRAPHIC CONDITIONS FOR SEPARATION OF CYCLIC DYNORPHIN A ANALOGUES FROM LINEAR BYPRODUCTS

INTRODUCTION AND LITERATURE REVIEW

The separation of a sample from impurities is critical to the success of an analysis. By definition, separation is the hypothetical condition where there is complete isolation, by *m* separate macroscopic regions, of each of the *m* chemical components which comprise a mixture. In other words, the goal of any separation process is to isolate the *m* chemical components into *m* vessels, such as glass vials or polyethylene bottles (28). The separation method must be rapid, reliable and reproducible.

Chromatography is a separation technique where one phase is held immobile (stationary phase) and the other phase (mobile phase) is passed over it (22). Key developments in chromatography are summarized in Table 1. Chromatographic separations are based on different rates of migration of solutes between the stationary phase and the mobile phase. Initially, a mixture is introduced into the system and carried by the mobile phase. Then, components in the mixture equilibrate or partition between the two phases, resulting in separation of the compounds (Figure 1). Different chromatography modes are classified based on the stationary phase and mobile phase (Table 2).

Table 1. Key developments in chromatography.

1906	Development of planar chromatography: thin layer chromatography (TLC) using silica gel as the planar support (17) Tswett (Russian Botanist) known as the Father of Chromatography described the separation of plant pigments by column liquid chromatography (4,8,22,25)
1940	Martin and Synge published their Noble Prize-winning paper about liquid-liquid (partition) chromatography and proposed the plate theory (25)
1950	Craig published a paper entitled "Partition Chromatography and Countercurrent Distribution" (6)
1952	Martin and Synge published a paper about gas chromatography for the separation of volatile fatty acids (21,22,25)
1955	Glueckauf published a paper entitled "The Plate Concept in Column Separation" (22) van Deemter, Zuiderweg and Klinkenberg proposed rate theory, the van Deemter plot, described chromatographic processes in terms of kinetics, and examined diffusion and mass transfer (32)
1963	Giddings published a paper entitled "Liquid Chromatography with Operating Conditions Analogous to Those of Gas Chromatography" (10) High performance liquid chromatography (HPLC) was developed and has been extensively used since then (10,22,31)

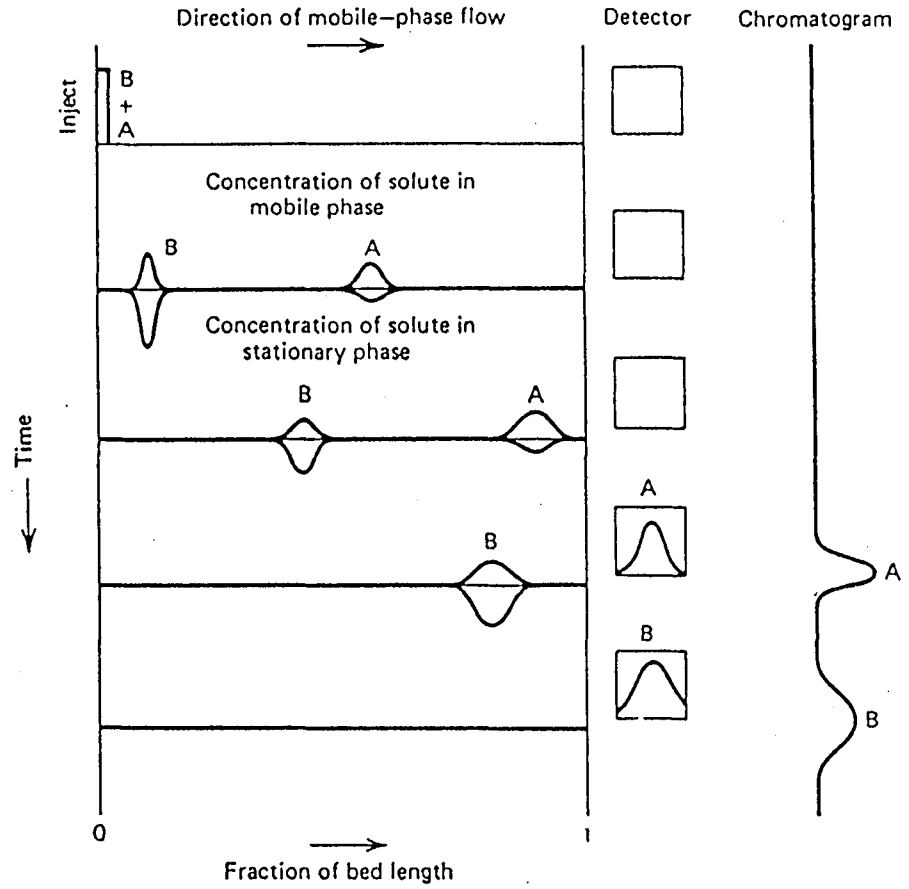
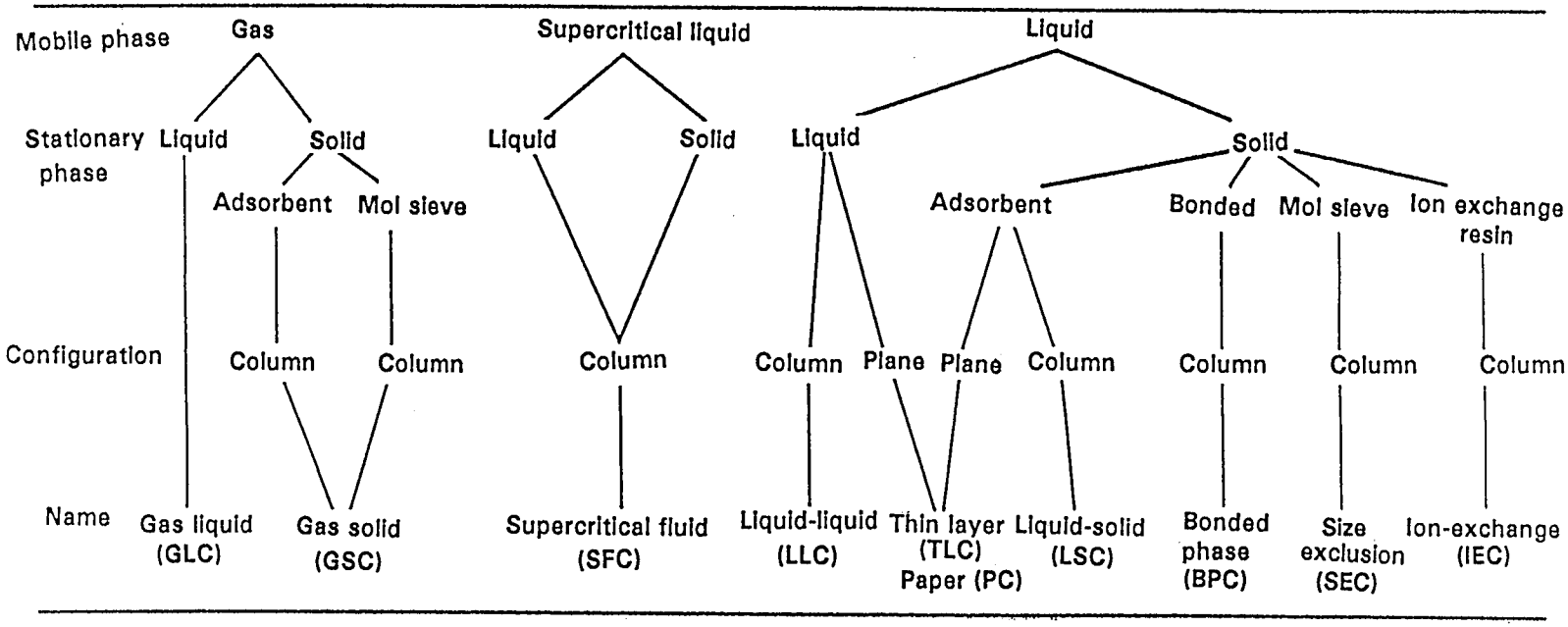


Figure 1. Schematic representation of the chromatographic process (22).

Table 2. Classification of chromatographic techniques (22).



High performance liquid chromatography (HPLC)

High performance liquid chromatography (HPLC) or modern liquid chromatography (LC) is an analytical technique that has been used since the late 1960's (Table 1). The main operational requirement of HPLC is the use of high pressure to enable the rapid diffusion rate of sample through the small uniform packing particle which results in fast analysis times (11). Figure 2 shows the basic components of an HPLC system. A single pump is sufficient for an isocratic analysis where the composition of mobile phase is constant. On the other hand, to perform a gradient analysis an additional pump is needed to pump a different solvent into a mixing chamber in order to vary the mobile phase composition (25).

Classical LC and HPLC are compared in Figure 3, and the differences between these two techniques are discussed below:

Classical LC and HPLC (30)

In classical LC a column is used only once, and therefore packing a column (step 1, bed preparation) has to be repeated. Sample application (step 2) requires skill and time on the part of the operator. Solvent flow (step 3) is achieved by gravity, and detection and quantitation (step 4) are achieved by the manual analysis of individual fractions.

Many separations, however, can be carried out on a given HPLC column since a closed, reusable column is used (step 1). Sample application (step 2) can be precisely performed without difficulty either by syringe injection or sample loop. Solvent flow (step 3) is achieved by using high pressure pumps, which give controlled and rapid flow of solvent resulting in reproducible operation with excellent accuracy. Detection and quantitation (step 4) are performed by an on-line detector which provides continuous chromatograms without intervention by an operator. Thus an accurate record of a separation can be obtained with minimum effort.

Classical LC is principally a preparative technique, while HPLC can be used for both analytical and preparative separations. The major advantages of HPLC over classical LC are the high speed, the enhancement of resolution and the high sensitivity of detection methods. HPLC, however, is much more expensive than classical LC which often limits the size of a purchased column and therefore limits the scale of a sample separation.

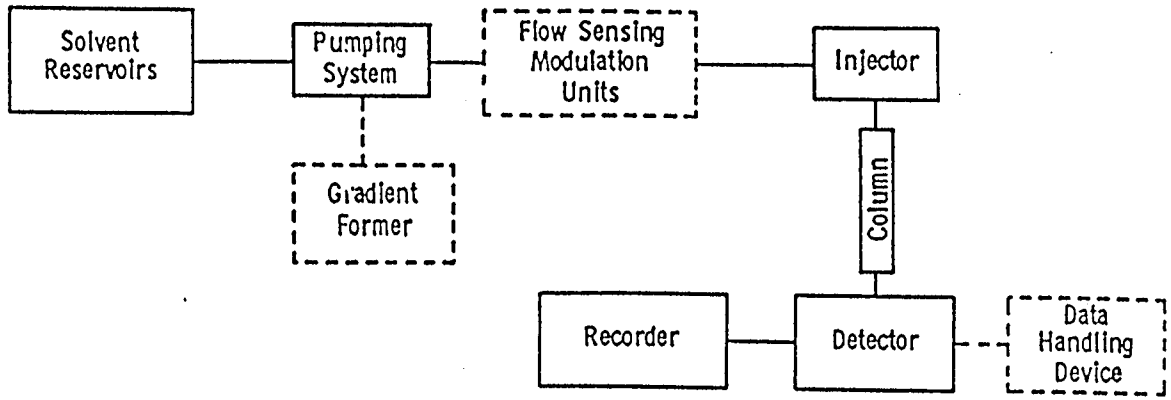


Figure 2. Block diagram of high performance liquid chromatography (25). Dotted lines refer to optional components.

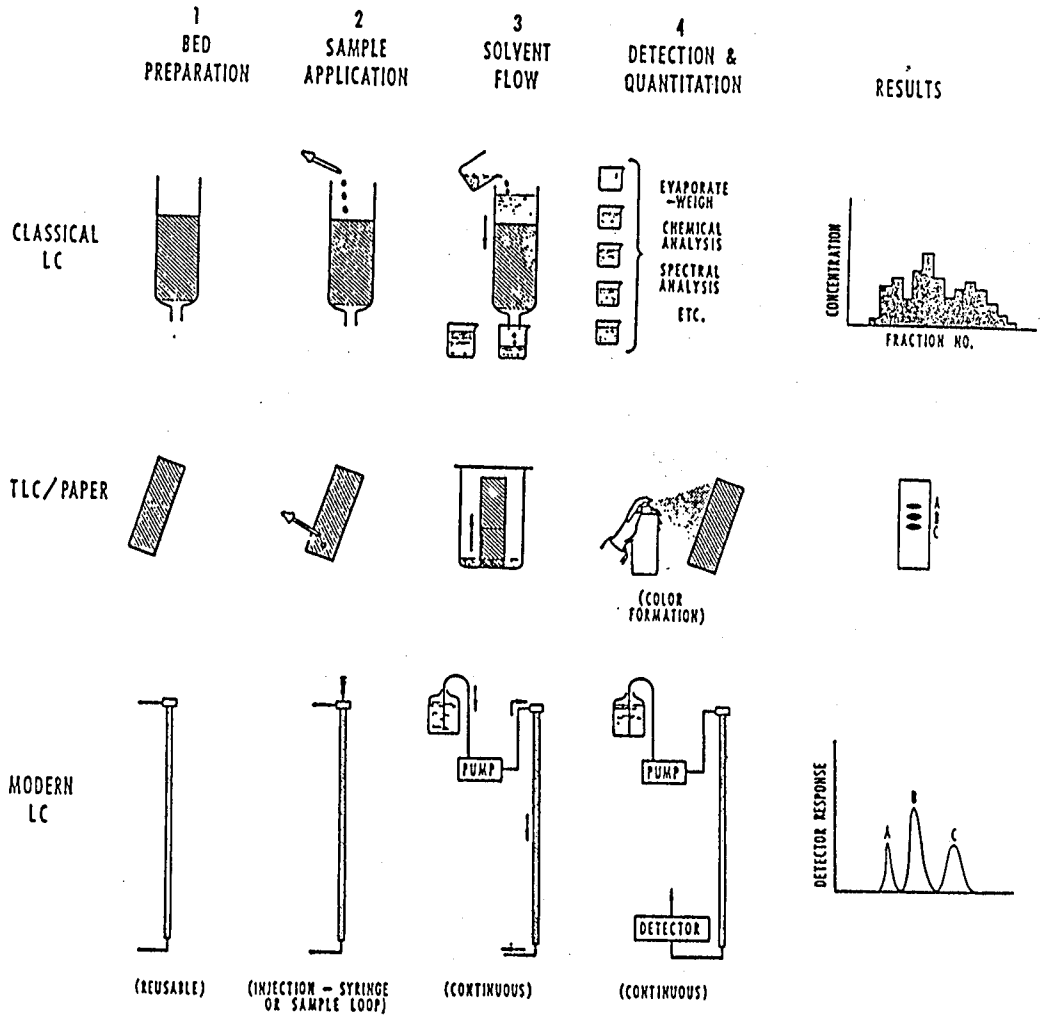


Figure 3. Different forms of liquid chromatography (30).

High performance liquid chromatography of peptides

The application of HPLC to peptide purification has been successfully employed for years since the method is very effective and reliable (7,13,20). The optimum conditions for purification, however, depend on an individual peptide's characteristics, including polarity, basicity, and molecular weight, and many factors can affect the separation of the desired peptides from impurities. First, the type of chromatography must be chosen. The three main modes of HPLC used for peptide separations utilize differences in peptide size (size exclusion HPLC or SEC), net charge (ion-exchange HPLC or IEC) and hydrophobicity (reverse phase HPLC or RPC) (Figure 4). In this study, only RPC and IEC are discussed in detail since the sizes of the desired peptides and the byproducts are similar.

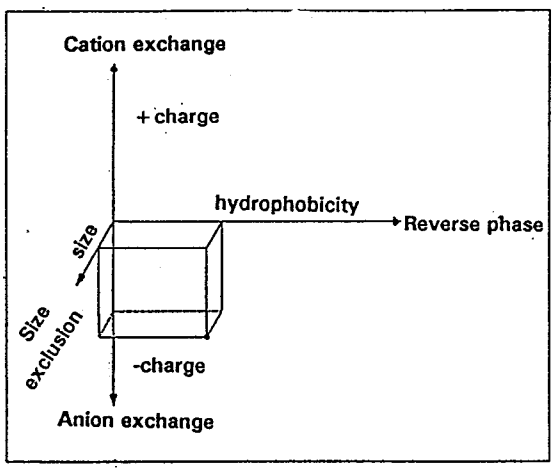


Figure 4. Schematic illustration of orthogonality in chromatographic separations (19).

Second, the stationary phase, which can vary in composition, particle size and pore size, has to be chosen. Finally, the mobile phase composition, including organic modifier, ion-pairing agent and pH, are examined to obtain optimum conditions for separation.

Reverse phase chromatography (RPC)

Reverse phase chromatography is a form of partition chromatography where the chemically bonded-phase (stationary phase) is hydrophobic or non-polar (e.g. octadecyl groups) and the starting mobile phase (e.g. water) is more polar. Separations by RPC involve differences in solute hydrophobicities. The most polar molecules will have the weakest interactions with the hydrophobic stationary phase, and thus have the smallest retention volumes and shortest retention times (17).

Peptides are comprised of amino acids which contain both hydrophilic (e.g. $-NH_3^+$, $-COO^-$ and -OH) and hydrophobic (e.g. aliphatic or aromatic hydrocarbon side chains) groups. Reverse phase chromatography with an aqueous mobile phase is appropriate to purify peptide mixtures since peptides are retained essentially according to their hydrophobic character. During separation, peptides exhibit regular reverse phase behaviors with a water-rich eluent of low pH and low ionic strength. Peptides are initially adsorbed on to the stationary phase and subsequently solvated by the solvent. Peptides remain on the support until there is a high enough concentration of organic modifier to displace or desorb the peptides from the support. This is the adsorption/displacement mechanism of interaction of peptides with a hydrophobic reverse-phase surface.

The commonly used columns in RPC contain a silica-based support (Figure 5), which can be functionalized by attachment of hydrocarbon chains to the hydroxyl side chains. Four media for RPC columns and their applications for peptide separations are summarized in Table 3.

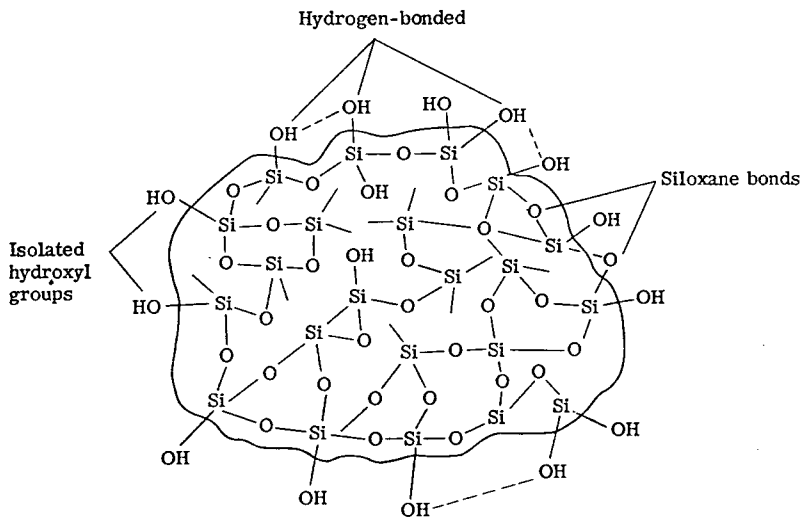


Figure 5. Structure of silica-based support (16).

Organic solvents used in the mobile phase for RPC must be chemically and physically compatible with the solute, the material used in the column support, the solvent delivery system and the detector. Mobile phase of low viscosity is favored since it can be run at a wide range of flow rates without high backpressure. Methanol, acetonitrile and 1- or 2-propanol are popular solvents used for peptide separations, although other water-miscible, UV-transparent solvents (e.g. methoxyethanol, ethanol, butanol,

tetrahydrofuran or dioxane) are also employed (7). Ion-pairing agents (e.g. trifluoroacetic acid (TFA), heptafluorobutyric acid (HFBA) or phosphoric acid (H_3PO_4)) are usually added to the mobile phase to displace peptides from the stationary phase.

The mobile phase pH has significant effects on peptide retention, since retention is dependent on the ionization states of free amino and carboxyl groups. Hydrophobic or non-polar peptides are retained on the hydrophobic surface of RPC; therefore a change in pH that increases the ionization of a peptide will enhance its polarity and shorten its retention time. On the other hand, decreasing the ionization state of a peptide reduces peptide polarity and lengthens its retention time.

Table 3. Media for RPC columns (14).

Media	Applications
C_4	For large peptides (greater than 20-30 residues), CNBr fragments and hydrophobic polypeptides
C_8	Peptide maps, natural and synthetic peptides and small hydrophilic protein
C_{18}	Enzymatic maps and small peptides
Diphenyl	Polypeptides containing aromatic sidechains

Ion-exchange chromatography (IEC)

Reverse phase chromatography is a widely used technique for purification of peptides, but there are cases where RPC is impractical. These include the separation of peptides which are extremely hydrophobic or hydrophilic and peptides which contain many charged amino acids (e.g. aspartic acid, glutamic acid, arginine, lysine and histidine). The very hydrophobic peptides are retained on non-polar surface of RPC, resulting in unreasonably long retention times, while hydrophilic or charged peptides are too polar to be held by the RPC stationary phase. In both cases, separation of the peptides cannot be achieved and aggregation of these peptides in the mobile phase may occur due to hydrophobic or electrostatic interactions (1).

Ion-exchange chromatography can be employed when RPC is not applicable, since separations by IEC are based on differences in peptide net charge. Anion-exchange (AEX) is useful for peptides containing acid residues (e.g. aspartic and glutamic acid), whereas cation-exchange (CEX) is useful for peptides containing basic residues (e.g. arginine, lysine and histidine) (20).

Stationary phases of IEC consist of bound ions and oppositely charged counter-ions (Figure 6); ionized solutes are retained by displacing the counterions. Peptide separations occur as a result of differences in the relative affinity of the peptide for the stationary phase ions as compared to the affinity of the mobile phase counterions in a system involving dynamic exchange (25,31). Electrostatic attraction is a major force between mobile phase ions, both charged peptides and eluents, and the ion centers on the

stationary phase.

IEC stationary phases or resins are composed of either silica or a polymer (e.g. divinylbenzene in a polystyrene matric) containing different functional groups, depending upon the type of exchanged ion (Table 4). Silica-based supports are rigid but readily dissolve at high pH (pH > 8.0), while polymeric supports possess greater pH stability (15,23).

The solvents used in the IEC mobile phase must meet the same requirements as those used in RPC mobile phases, including compatibility with solutes and the HPLC system, low viscosity and UV transparency. The most widely used solvents are phosphate or acetate buffers. Salt counter-ions are added to displace solute ions from the charged sites on the stationary phase. Different salts exhibit distinct effects on peptide retentions; divalent salts are stronger displacers than monovalent salts, and smaller ions are also stronger than larger ions. The order of decreasing elution strengths of commonly used salts are (23):

cations: $Mg^{2+} > Ca^{2+} > NH_4^+ > Na^+ > K^+$

anion: $SO_4^{2-} \geq HPO_4^{2-} > Cl^- > CH_3COO^-$

Amino acids are amphoteric molecules containing both positive and negative charges. Therefore, manipulating the pH of the mobile phase will alter the net charge of peptides. The pH of the mobile phase should be at least 0.5 pH units above the isoelectric point (pI) of the peptide for AEX and below the pI for CEX (23).

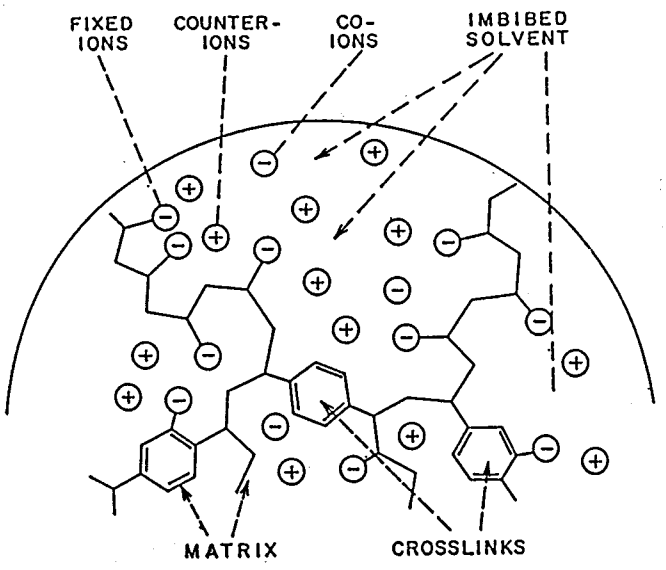


Figure 6. Structure of an ion-exchange resin (25).

Table 4. Common types of ion-exchange resins (2).

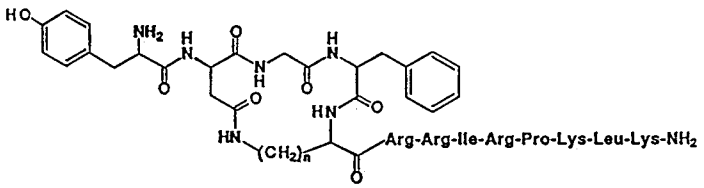
	Cation		Anion	
	Strongly acidic	Weakly acidic	Strongly basic	Weakly basic
Functional groups	$-SO_3H$	-COOH	$-N^+(CH_3)_3Cl^-$	$-N^+R_2HCl^-$

OBJECTIVES

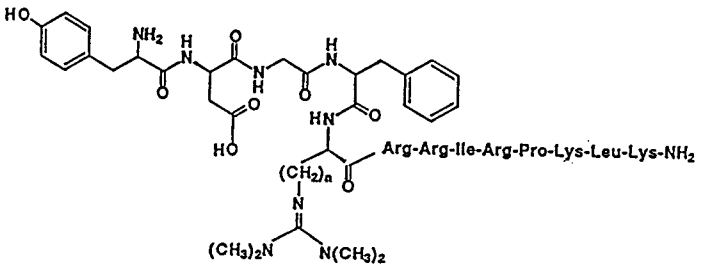
Research in our laboratory involves preparing analogues of the opioid peptide dynorphin A, which is thought to be an endogenous ligand for κ opioid receptors (5). Constrained cyclic analogues are being prepared to explore possible biologically active conformations at these receptors. During the preparation of one series of cyclic analogues, cyclo[D-Asp^2,X^5]Dyn A (1-13)NH_2, where X is ornithine (Orn), α,γ-diaminobutyric acid (Dab) or α, β-diaminopropinoic acid (Dap) (Figure 7, (I)), different side products were obtained depending upon the activation reagent used for the cyclization (Arttamangkul and Aldrich, manuscript in preparation). The tetramethylguanidinium (Tmg) derivative, [D-Asp^2,X (Tmg)^5]Dyn A(1-13)NH_2 (Figure 7, (II)) was the major side product when 2-(1-H-benzotriazo-1-yl)-1,1,3,3 tetramethyluronium hexafluorophosphate (HBTU) (9,18) was used, whereas the linear peptide, [D-Asp^2,X^5]Dyn A (1-13)NH_2 (Figure 7, (III)), was obtained as the only side product when other reagents (benzotriazolyl-N-oxytridimethylaminophosphonium hexafluoro-phosphate (BOP)(29) or diisopropyl carbodiimide (29)) were used. The unusual Tmg derivatives resulted from transferring of the tetramethyluronium group from HBTU to the side chain amine.

The following study focuses on using high performance liquid chromatography (HPLC) to separate the cyclic dynorphin A analogues, cyclo[D-Asp^2,X^5]Dyn A (1-13)NH_2 from the Tmg side product. Two modes of HPLC, reverse phase (RPC) and ion-exchange (IEC) chromatography, were examined under various conditions utilizing different pHs,

organic modifiers and ion-pairing agents. These factors were varied to obtain optimum conditions for purification of the cyclic peptides on a preparative scale. All results from the separation of ornithine analogue are discussed and selected results for other analogues (diaminobutyric acid and diaminopropionic acid) are shown in the appendices.



(I)



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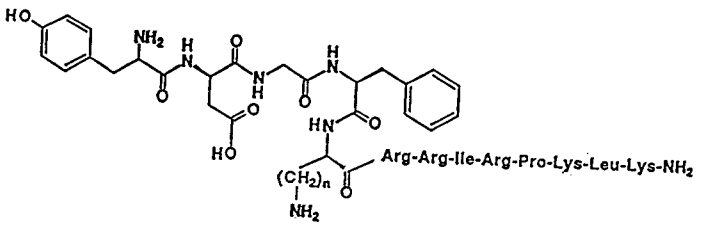


(III)

Figure 7. Structure of cyclo[D-Asp^2,X^5]Dyn A (1-13)NH_2 (I), [D-Asp^2,X(Tmg)^5]Dyn A (1-13)NH_2 (II) and [D-Asp^2,X^5]Dyn A (1-13)NH_2 (III) analogues.

EXPERIMENTAL METHODS

Equipment and materials

The HPLC system consisted of two Beckman 110B pumps, a 421A controller, a 163 variable wavelength detector model 334, injector with a 20 μL or 500 μL (for the loading studies) loop, and a Waters 740 data module integrator. Eluents were monitored at 214 nm for mobile phase systems 1, 4-6 and 8 and at 225 nm for systems 2 and 7 (Table 5). Loading studies were monitored at 280 nm. Separations were carried out on three reverse phase columns and one ion exchange column. The Vydac column (The Separation Group, Hespernia, CA), Zorbax Protein Plus column (DuPont, Wilmington, DE), and Dynamax column (Rainin, Emeryville, CA) are reverse phase columns containing spherical butyl-bonded (C_4) silica as the stationary phase. The strong cation exchange (SCX) column (The Nest Group, Southborough, MA) consists of polysulfoethyl aspartamide polymeric surfaces (Figure 8). For all of the columns the dimensions were 4.6 x 25 mm except for the SCX guard cartridge, which was 4.6 x 50 mm and the stationary phase had a 5 μm particle size (except for the Zorbax column, which was 10 μm) and 300 Å pore size.

Solvents and reagents used for HPLC were obtained from the following sources: acetonitrile-UV and methanol (Burdick & Jackson, Muskegon, MI); Ionate™ trifluoroacetic acid (TFA), heptafluorobutyric acid (HBFA) and triethylamine (TEA) (Pierce Chemical Co., Rockville, IL). Other reagents were reagent grade: KCl and

KH_2PO_4 (Mallinckrodt, Paris, KY); H_3PO_4 (J T Baker Chemical, Phillipsburg, NJ). Water was obtained from a Millipore Mill Q System (College of Pharmacy) and all mobile phases were filtered through a 0.45 μm Nylaflo membrane (Gelman Science, Ann Arbor, MI) and degassed before used.

Procedure

Peptides were synthesized as described elsewhere (Arttamangkul and Aldrich, manuscript in preparation). Crude peptides A, B and C in Table 6 (structures shown in Figure 7), and pure cyclic, Tmg and linear derivatives of each peptide were dissolved in water to give a concentration of 1 mg/mL. HPLC analyses were performed on reverse phase columns using a standard gradient of 0%-75% of solvent B (Table 5) over 50 min with flow a rate of 1.5 mL/min. The effects of ion-pairing agents and organic modifiers were studied by using the mobile phase systems described in Table 6. The triethylammonium phosphate (TEAP) buffers I and II were obtained by bringing the pH of 0.25 N and 0.09 N phosphoric acid solutions, respectively, to 2.5 with triethylamine (27). A loading study of 1 and 3 mg of crude peptide A was investigated on Zorbax column using TEAP II buffer. Method development for separation on the SCX column was done on a guard cartridge (4.6 x 50 mm) by varying the percent of acetonitrile and the pH of the mobile phase. The best conditions were chosen to separate the crude peptides on the analytical column. The loading capacity of the analytical column was also studied using 1, 5 and 10 mg of crude peptide A.

Table 5. Composition of the mobile phases for HPLC[1, 2]

System	Solvent A	Solvent B
1	0.1% TFA in H_2O	0.1% TFA in AcCN
2	0.1% TFA in H_2O	0.1% TFA in MeOH
3	0.1% HFBA in H_2O	0.1% HFBA in AcCN
4	0.1% H_3PO_4 in H_2O	0.1% H_3PO_4 in AcCN
5	TEAP(I)	AcCN
6	TEAP(II)	40%A + 60% AcCN
7	TEAP(I)	MeOH
8	5 mM KH_2PO_4 in x% AcCN	A + 800 mM KCl

[1]Systems 1 through 7 were used for reverse phase HPLC and system 8 was used for ion-exchange chromatography.

[2]TFA = trifluoroacetic acid; HFBA = heptafluorobutyric acid; TEAP(I) = triethylammonium phosphate buffer (0.25 N H_3PO_4 in TEA/H_2O); TEAP(II) = triethylammonium phosphate buffer (0.09 N H_3PO_4 in TEA/H_2O); TEA = triethylamine; x% = 10%, 20% or 35%

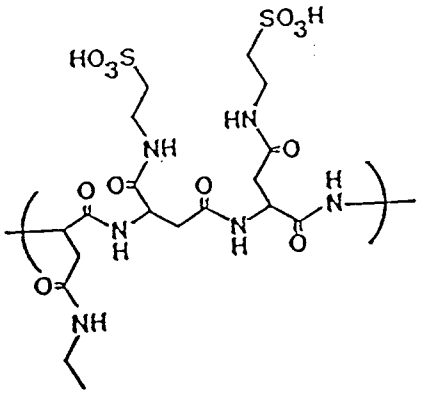


Figure 8. Stationary phase of SCX column (polysulfoethyl aspartamide) (1).

Table 6. Synthetic peptides studied.

Crude peptide	Composition
A	cyclic and Tmg [D-Asp^2,Orn^5]Dyn A (1-13)NH_2
B	cyclic and Tmg [D-Asp^2,Dab^5]Dyn A (1-13)NH_2
C	cyclic and Tmg [D-Asp^2,Dap^5]Dyn A (1-13)NH_2

Tmg = trimethylguanidinium; Dab = diaminobutyric acid; Dap = diaminopropionic acid.

Calculations

The degree of separation is defined as the resolution value (R_S) which is calculated from equation (a) when components in a sample are well resolved and there is no baseline drift (Figure 9a). Larger values of R_S mean better separation, and smaller values of R_S indicate poorer separation.

$$R_s = (t_2 - t_1)/0.5\ (W_2 + W_1) \qquad (a)$$

where t_1, t_2 = elution times and W_1, W_2 = baseline peak widths in minutes of the first and second peak, respectively, in the chromatogram (30) (Figure 9a).

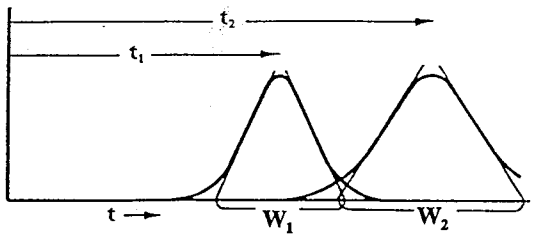
The separation of a real sample is usually complicated by baseline drift and minor impurities(Figure 9b), which can make it difficult to measure baseline peak widths. The more accurate R_S values are obtained by using equation (b) (23), where W_1 and W_2 are peak widths at half height in minutes (Figure 9b).

$$R_S = 1.18\ (t_2 - t_1)/(W_2 + W_1) \qquad (b)$$

Resolution values shown on the chromatograms and the discussion of the results in this study are based on calculations using equation (b). Comparisons of purified peptide retention times under the HPLC conditions used for separation of crude peptide A are shown in Tables 8.1 and 8.2. Tables 9.1 and 9.2 summarize the R_s values calculated from both equations (a) and (b) for the cyclic and Tmg peptides from separation of crude peptide A under various HPLC conditions.

a)

$$R_S = \frac{2\,(t_2 - t_1)}{(W_2 + W_1)}$$



b)

$$R_S = \frac{1.18\,(t_2 - t_1)}{(W_2 + W_1)}$$

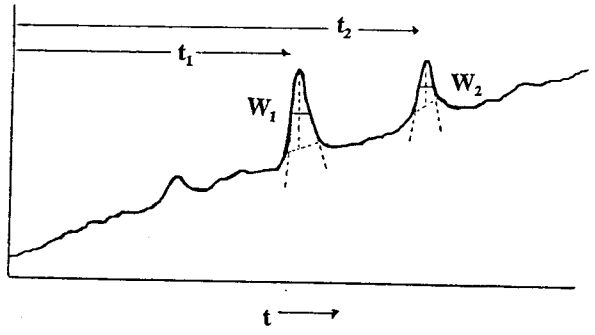


Figure 9. Resolution in HPLC: a) ideal sample and b) real sample (23,30).

RESULTS AND DISCUSSION

Reverse phase chromatography

Comparison by standard RPC condition (0.1% TFA in aqueous AcCN) of [D-Asp^2,Orn^5]Dyn A (1-13)NH_2 cyclized with HBTU indicated that the Tmg side product (t_R = 16.2 min) was more difficult to separate from the cyclic peptide (t_R = 18.2 min) (Figure 10a) than was the separation of the linear peptide (t_R = 14.8 min) (Table 8.1) from the cyclic peptide. Therefore, the separation of desired cyclic peptide from the Tmg byproduct was examined by RPC under various conditions. The effects of several ion-pairing agents, pH and different organic solvents (e.g. AcCN and MeOH) were studied.

Effect of ion-pairing agents

In RPC, peptides are initially adsorbed on hydrophobic surfaces of the stationary phase and subsequently desorbed by the organic solvent (12). Since they are typically charged molecules at pHs used in chromatography, different counterions will influence their chromatographic behavior, and changing the ion-pairing agent may improve resolution. Anionic counterions (e.g. TFA, HFBA and H_3PO_4) interact with ionized residues within the peptide and affect its retention. Ion-pairing agents such as TEAP and H_3PO_4 can be used, but a desalting step is required in the final purification process (26,27).

Although TFA is routinely used for peptide purification because of its volatility, efficiency and UV transparency at typical detection wavelengths (12,20), it was not an ideal agent to separate the peptides in our study when AcCN was used as the organic solvent (Figure 10a). Other ion-pairing agents were investigated with the following order of hydrophobicity : HFBA > TFA > H_3PO_4 (13). TEAP buffer and 0.1% H_3PO_4 solution gave similar resolutions, although TEAP buffer gave slightly longer retention times. HFBA, however, was an ineffective ion-pairing agent which could not separate the desired cyclic peptide from the Tmg byproduct (Figure 10b). Except when HFBA was used as the ion-pairing agent, the Tmg side product was eluted prior to the cyclic peptide (Figure 10 and Table 9.1). Since the Tmg side product is more polar than the cyclic peptide due to additional acidic and basic residues in the molecule (Figure 7), it was retained less by the hydrophobic RPC column.

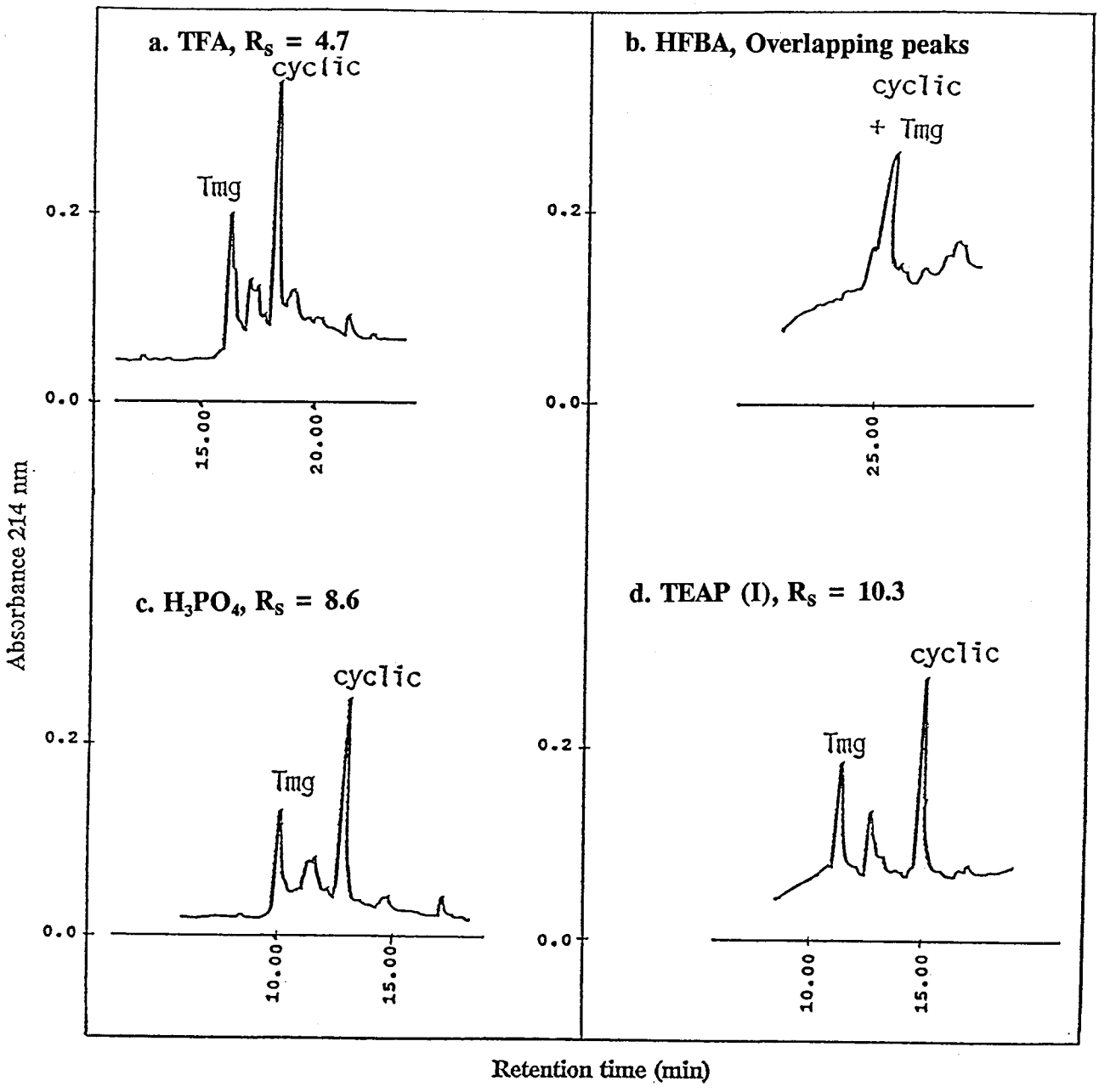


Figure 10. Effects of ion-pairing agents on RPC. Sample: crude peptide A. Column: Vydac. Mobile phase: solvent A = 0.1%x in H_2O, solvent B = 0.1%x in AcCN, where a) x = TFA, b) x = HFBA, c) x = H_3PO_4 and d) solvent A = TEAP(I), solvent B = AcCN. Flow rate: 1.5 mL/min. Gradient: 0% to 75% B over 50 min.

Effect of pH of the mobile phase

Changing the pH of the mobile phase can alter peptide resolution in RPC due to changes in the ionization state of amino acid residues. Most peptides can be separated at acidic or neutral pH (20). A lower pH (< 3.0) is more favorable than higher pH (> 8.0), since at high pH the silanol sites on the surface of the silica-based column are ionized, which results in poor reproducibility and shortening of column life (13,19,20,26). Comparison of the effects of a TEAP buffer at two different pHs, 2.25 and 5.0, on the elution profile of crude peptide A are shown in Figure 11 and R_s values are listed in Table 9.1. Increasing the pH to 5.0 adversely affected the separations.

Effect of organic modifiers

An organic modifier is a water-miscible solvent added to the aqueous solvent to affect separation of peptides. The separation of peptides by RPC is based on differences in hydrophobicity. Therefore the polarity of organic modifiers can alter the retention of peptides. Since using TFA in AcCN did not give the best resolution for our peptides, MeOH was examined as a possible organic solvent using both TFA and TEAP as ion-pairing agents.

The retention times of the cyclic dynorphin A analogue and the Tmg side product in MeOH are longer than in AcCN for both ion-pairing agents (TFA and TEAP) (Figures 12.1 and 12.2, and Table 9.1). The overall hydrophobicity and viscosity of MeOH is higher than AcCN (Table 7), and MeOH requires a longer time to elute the peptide from

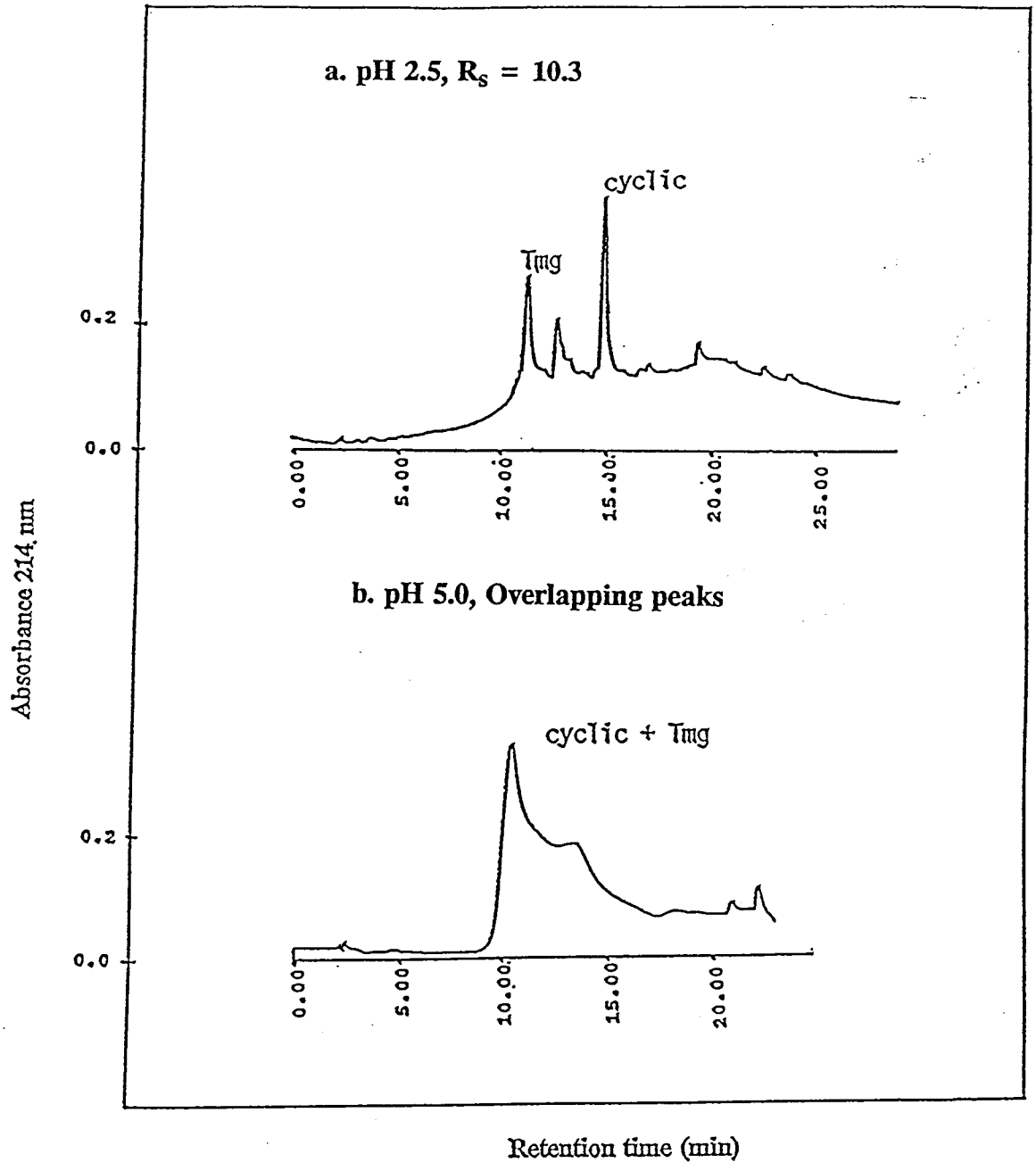


Figure 11. Effects of mobile phase pH on RPC. Sample: crude peptide A. Column: Vydac. Mobile phase: solvent A = TEAP(I) at a) pH 2.5 and b) pH 5 solvent B = AcCN. Flow rate: 1.5 mL/min. Gradient: 0% to 75% B over 50 min.

the non-polar surface of the RPC column. Since MeOH has a higher UV cutoff (210 nm) than AcCN (195 nm)(Table 7), the optimum absorption wavelength for peptides (214 nm) (24) cannot be used when MeOH is an organic modifier and the peak heights from the separation by MeOH are shorter than those from AcCN (Figure 12.1 and 12.2) Using TFA as the ion-pairing agent, MeOH gave a better separation and higher R_S than AcCN (Figure 12.1), however, TEAP (I) in AcCN provided superior results to TEAP (I) in MeOH (Figure 12.2).

Table 7. Properties of AcCN and MeOH (15)

	Dielectric constant	Polarity	Viscosity	UV cutoff[1]
AcCN	37.5	6.2	0.37	195 nm
MeOH	32.6	6.6	0.60	210 nm

[1]UV cutofff is the wavelength at which the solvent has an absorbance of 1.0 absorbance unit (AU).

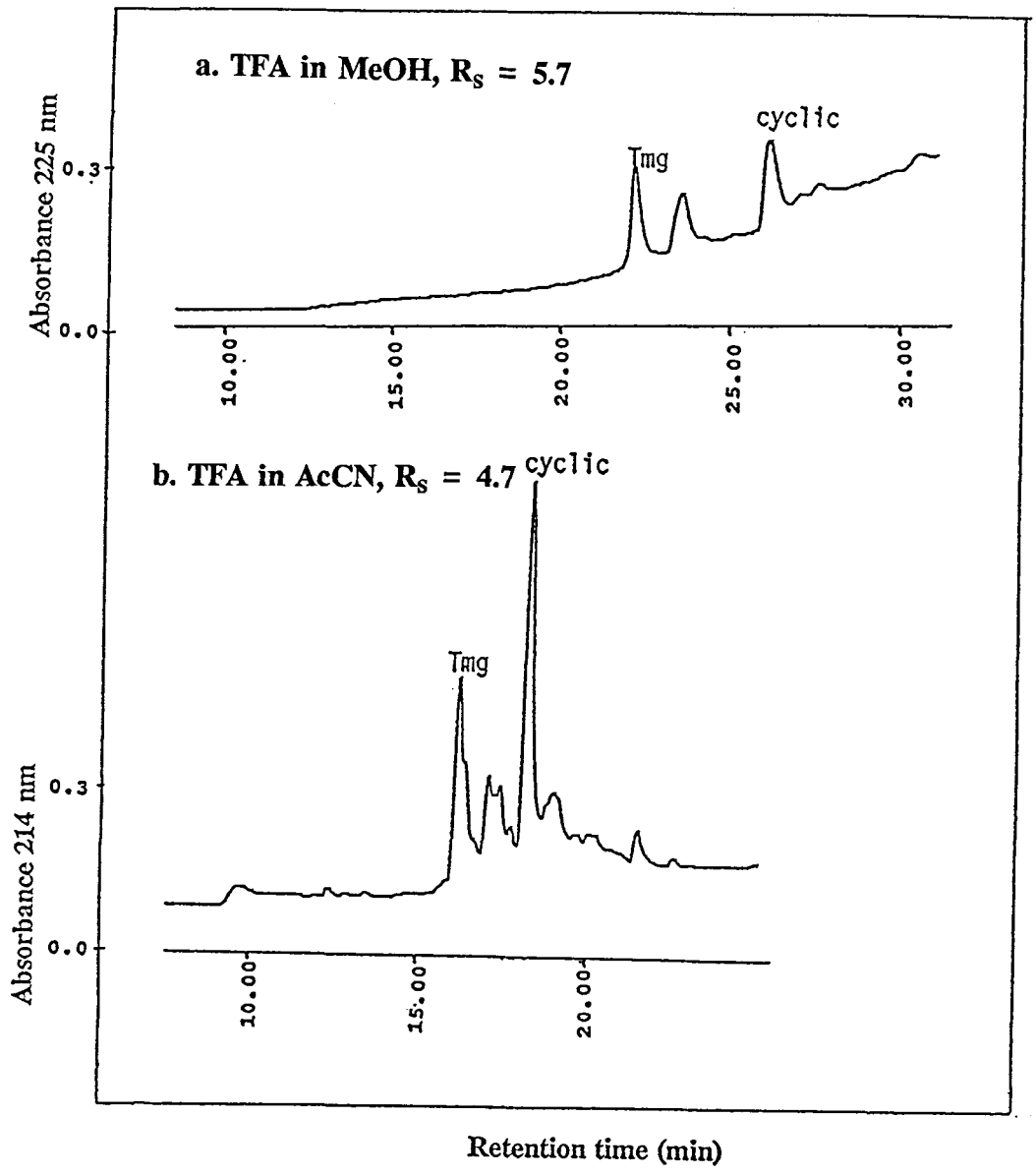


Figure 12.1. Effects of organic modifiers on RPC using TFA as ion-pairing agent. Sample: crude peptide A. Column: Vydac. Mobile phase: solvent A = 0.1% TFA in H_2O, solvent B = 0.1% TFA in a) MeOH and b) AcCN. Flow rate: 1.5 mL/min. Gradient: 0% to 75% B over 50 min.

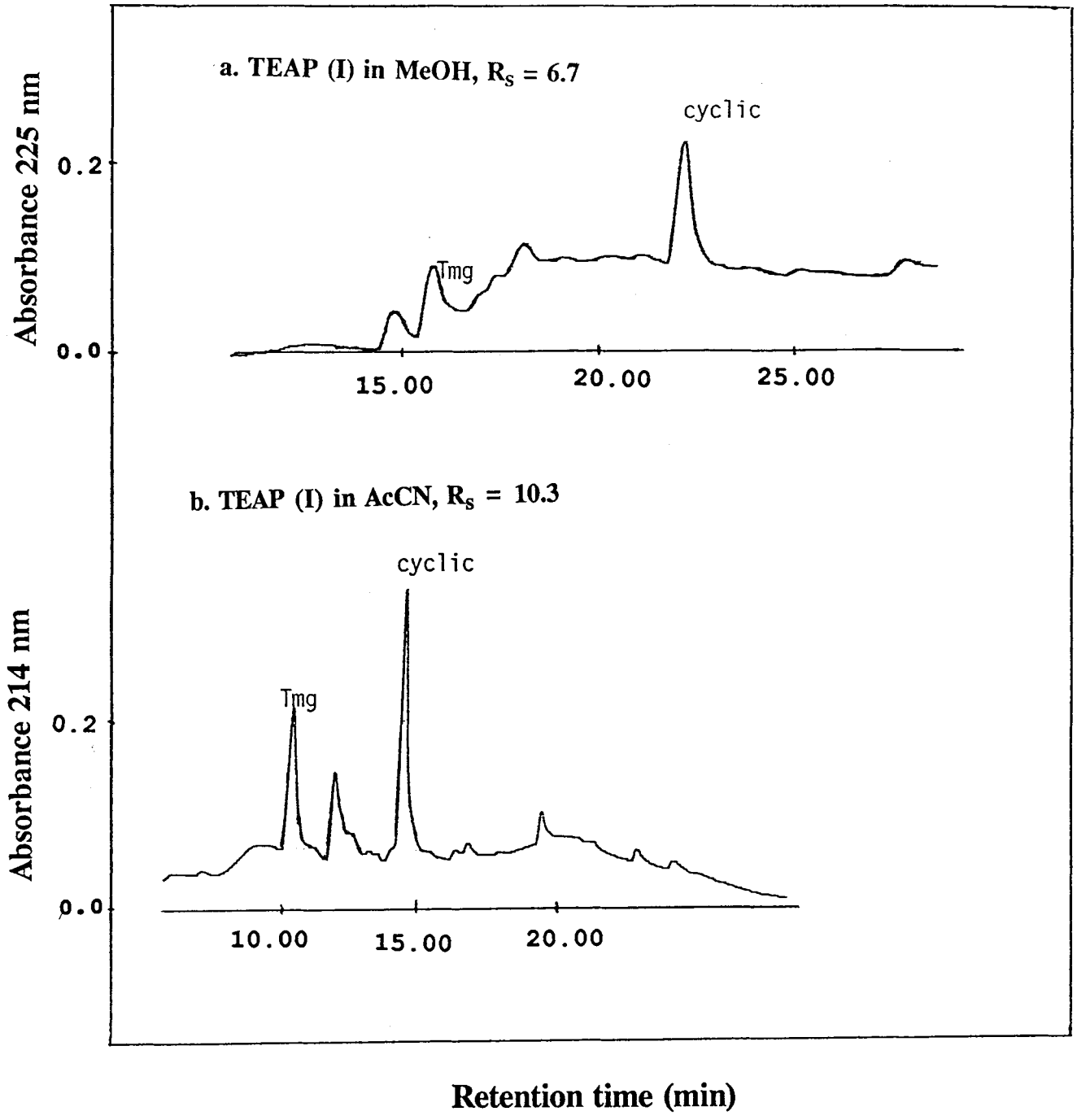


Figure 12.2. Effects of organic modifiers on RPC using TEAP as ion-pairing agent. Sample: crude peptide A. Column: Vydac. Mobile phase: solvent A = TEAP (I) pH 2.5, solvent B = a) MeOH and b) AcCN. Flow rate: 1.5 mL/min. Gradient: 0% to 75% B over 50 min.

Loading study on RPC

Resolution during peptide purifications depends on the amount of sample loaded on the column. An analytical column can be used to determine appropriate quantities that can be loaded during a preparative run. In order to determine how much material could be separated on a preparative column, a loading study of crude peptide A was performed. The Zorbax column was chosen since this column has the same stationary phase as the preparative column used in our laboratory. A lower concentration of TEAP (0.09 N) (TEAP (II)) was used instead of TEAP (I) (0.25 N TEAP) in order to reduce the amounts of phosphate salt generated in the mobile phase (27). A slower gradient of AcCN was employed in TEAP II. TEAP II provided a smaller R_S value than TEAP I, however, the cyclic peptide was better separated from the Tmg byproduct (Figure 13.1). The chromatogram for an analytical sample (20 μg) of crude peptide A mixture separated by using TEAP (II) on the Zorbax column is shown in Figure 13.2.

The analytical Zorbax column (4.6 mm ID) could separate the desired cyclic peptide from the Tmg byproduct using 1 mg of crude peptide A (Figure 13.3a) which is equivalent to 20 mg on the one inch diameter preparative column. The resolution decreased some when loading of the column was increased to 3 mg of the crude mixture (Figure 13.3b), but a reasonable separation was still obtained. Therefore, the maximum capacity of the one inch diameter preparative column using these conditions is 50-60 mg.

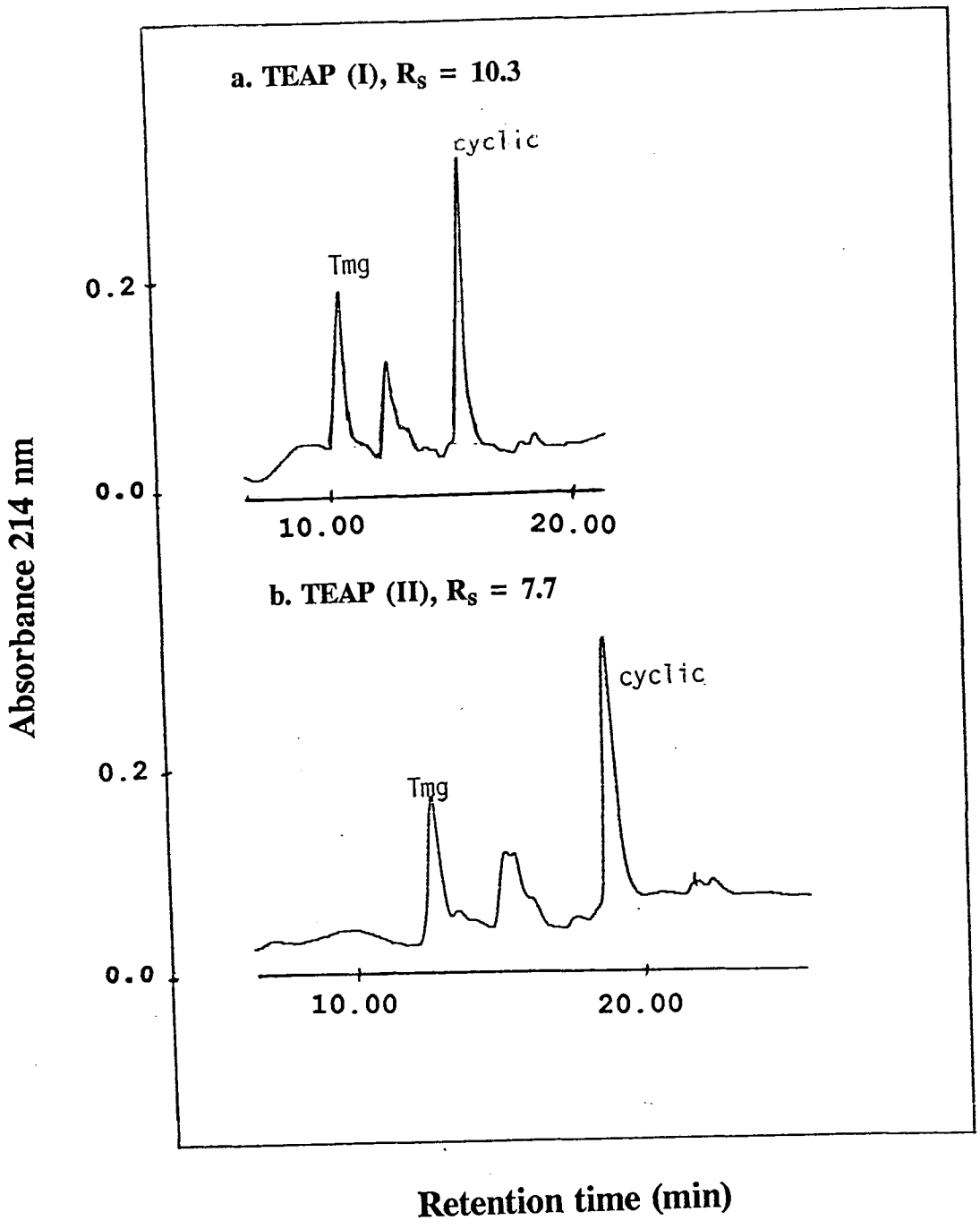


Figure 13.1. Effects of concentration of TEAP and AcCN on RPC. Sample: crude peptide A. Column: Vydac. Mobile phase: solvent A = a) TEAP (I)and b) TEAP (II) pH 2.5, solvent B = a) AcCN and b) 40% A + 60% AcCN. Flow rate: 1.5 mL/min. Gradient: 0% to 75% B over 50 min.

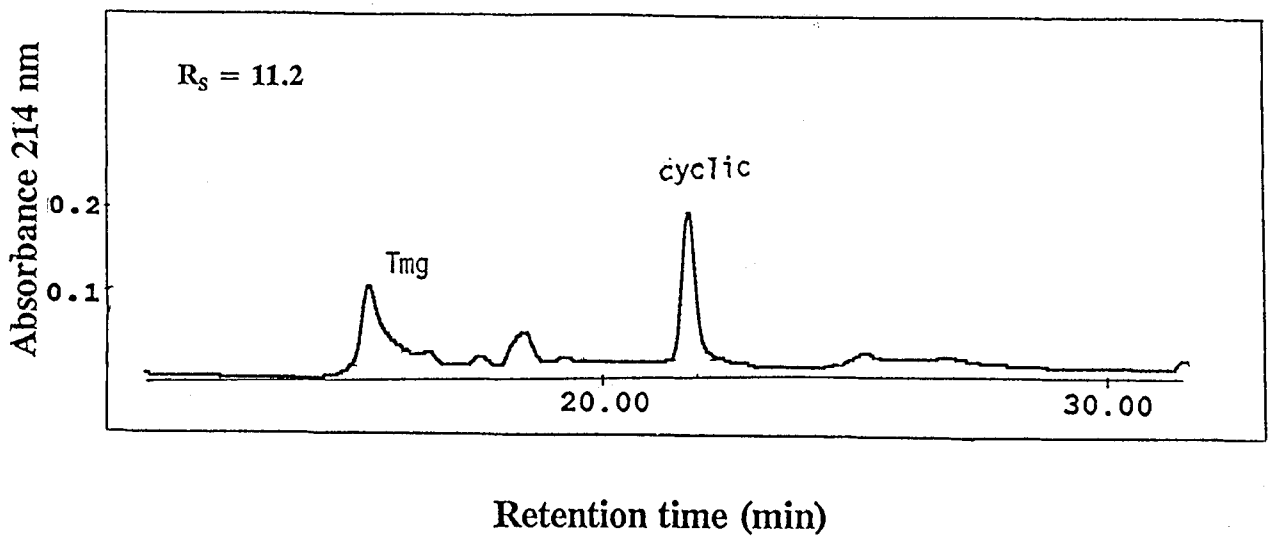


Figure 13.2. A preliminary run of crude peptide A mixture (20 µg) on the Zorbax column. Mobile phase: solvent A = TEAP (II) pH 2.5, solvent B = 40% A + 60% AcCN. Flow rate: 1.5 mL/min. Gradient: 0% to 75% B over 50 min.

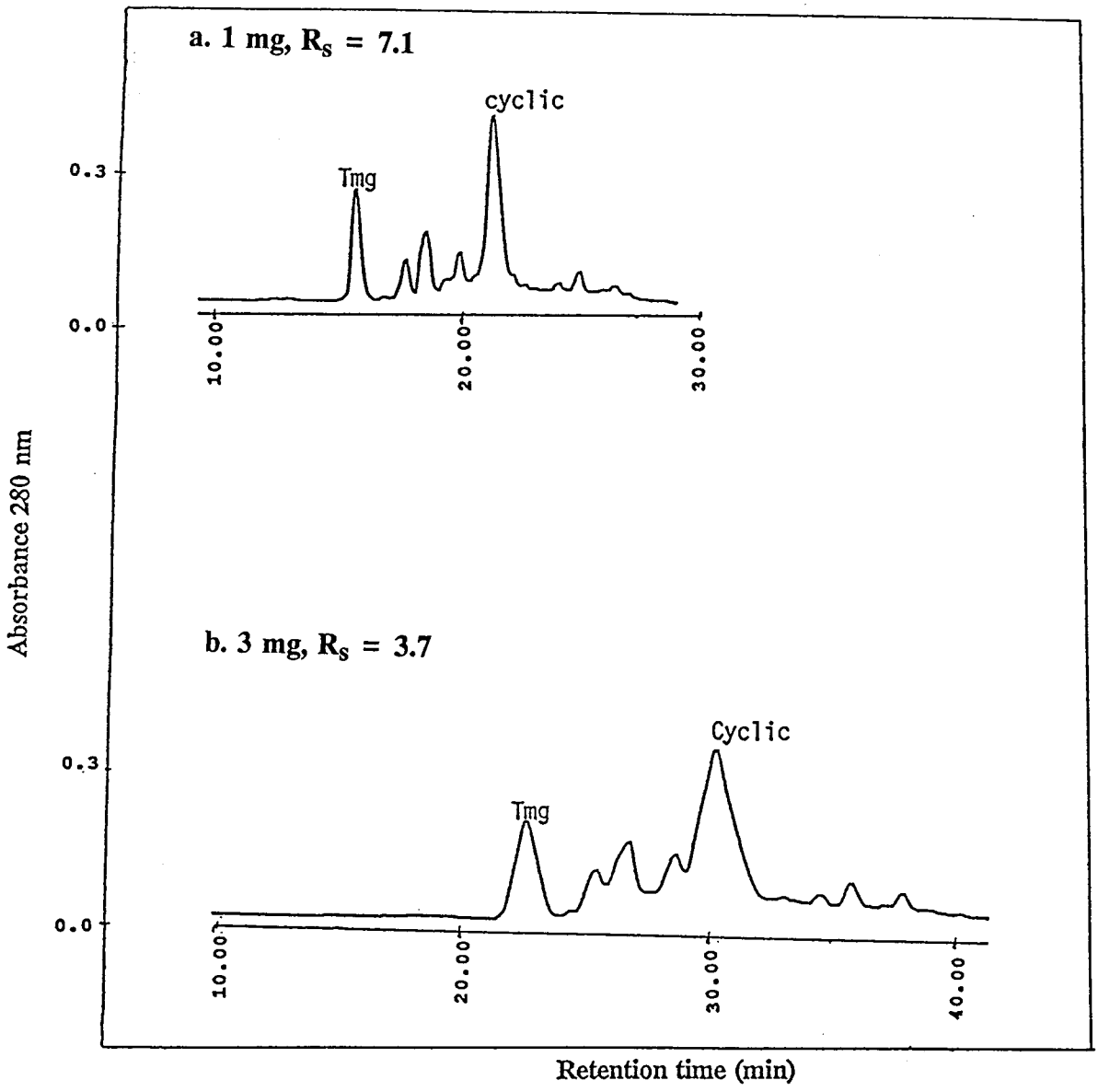


Figure 13.3. Loading studies on RPC. Sample: crude peptide A a) 1 mg and b) 3 mg. Column: Zorbax Pro. Mobile phase: solvent A = TEAP(II) pH 2.5, solvent B = solvent A + 60% AcCN. Flow rate: 1.5 mL/min. Gradient: 0% to 75% B over 50 min.

Ion exchange chromatography

IEC is an alternative mode for separation of peptides containing highly charged acidic or basic amino acids. The cyclic dynorphin A analogues in this study carry positive charges from the N-terminus and five basic amino acids at positions 6, 7, 9, 11 and 13 (Figure 7), whereas the Tmg and linear byproducts have one acidic residue and one additional basic residue. The SCX column containing polysulfoethyl aspartamide as the stationary phase (Figure 8) resolves peptides by the ability of sulfonate groups to retain positively charged peptides at neutral or lower pH (1). Therefore, ion exchange chromatography using SCX column is a logical method for resolving the cyclic peptides from the Tmg byproducts in the crude peptide mixtures. The separation of the cyclic peptide from the linear byproducts using ion exchange chromatography was examined using a phosphate buffer containing AcCN with a gradient of KCl to elute the peptides. Method development using different pHs and organic solvents for separation on the SCX column was done on a guard cartridge (4.6 x 50 mm) rather than on the analytical column to reduce analysis time.

Effect of pH of the mobile phase

Ion exchange chromatography separates peptides by utilizing differences in their net charge. The expression of the peptide's net charge depends on the pH, e.g. in a basic or neutral mobile phase N-terminal and side chain amino groups are not fully charged,

while they are positively charged in an acidic solvent. Therefore it is apparent that pH could have a large effect on the separation of the cyclic peptides from the Tmg byproducts.

The ionization state of the cyclic peptide is the same at neutral or acidic pHs since the D-aspartic acid residue is cyclized to give the lactam ring. At lower pH (3.0) the D-aspartic acid side chain (pK_a = 3.9) of the Tmg byproduct, however, is predominately in the neutral form, while at higher pH (5.0) this residue is deprotonated. The net charge of the cyclic peptide is +6 at both pHs (3.0 and 5.0), whereas, the charge of the Tmg byproduct is +7 and +6 at pH 3.0 and 5.0, respectively. Thus the difference in the net charge of the desired cyclic peptide (+6) and the Tmg byproduct (+7) at pH 3.0 should enhance the separation.

The cyclic peptide was eluted from the SCX column prior to the Tmg byproduct at pH 3.0 (Figure 14) since the cyclic peptide has a lower net charge (+6) than the Tmg byproduct (+7), while at pH 5.0 overlapping peaks were obtained. Therefore, pH 3.0 was chosen for use in further experiments in this study.

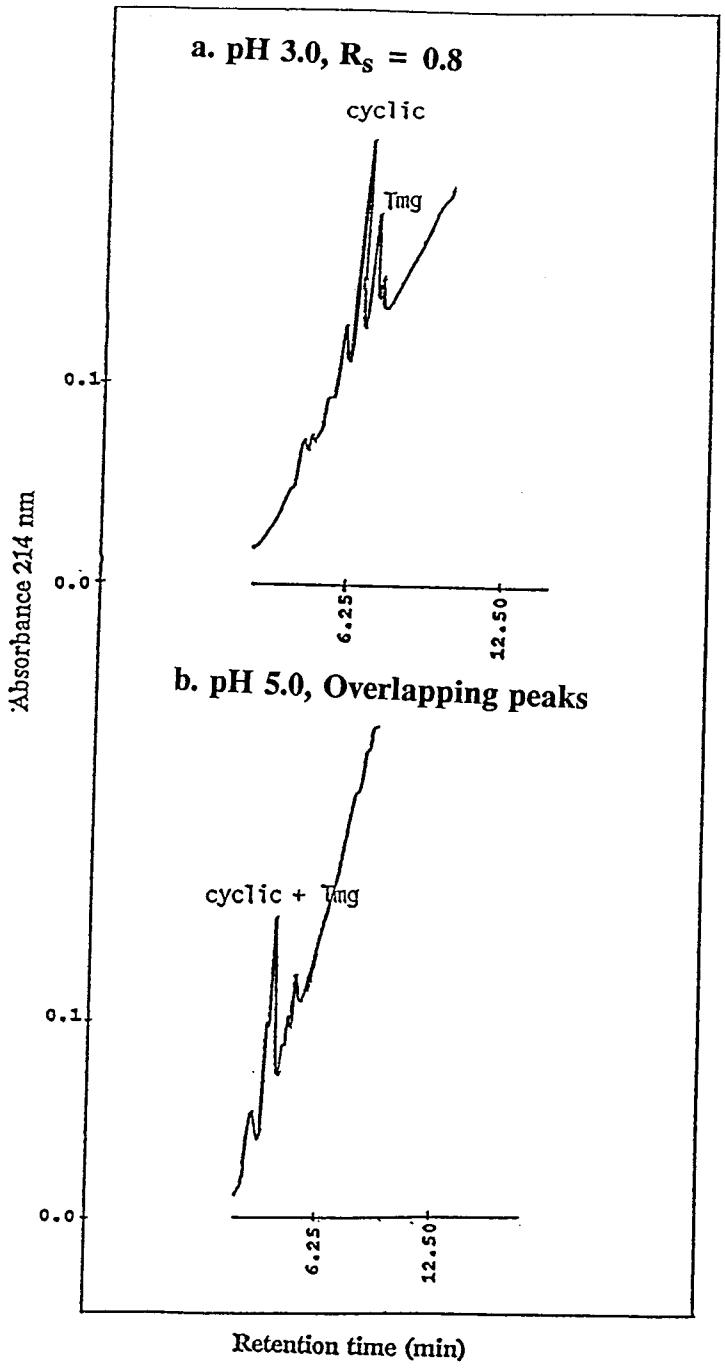


Figure 14. Effects of mobile phase pH on IEC. Sample: crude peptide A. Column: SCX guard cartridge. Mobile phase: buffer A = 5 mM KH_2PO_4+25% AcCN at a) pH 3.0 and b) pH 5.0, solvent B = solvent A + 800 mM KCl. Flow rate: 1.5 mL/min. Gradient: 10% to 70% B over 12 min.

Effect of organic modifiers

The mechanism of IEC is primarily electrostatic interactions between the solute and the ion-exchange packing. The stationary phase exhibits some hydrophobic characteristics, however, which results in poor peak shape and poor resolution (3). A small amount of organic solvent, e.g. 10-15% of AcCN, in the mobile phase is recommended to suppress the hydrophobicity of the IEC packing (3). A higher concentration of AcCN (> 50%) promotes mixed-mode hydrophilic and ionic interactions between peptides and the stationary phase, which can be useful for the separation of peptides containing the same net charge (1,3). The resolution of peptides, however, may either improve or decrease depending on the properties of each peptide. For example in 5 mM potassium phosphate buffer at pH 3.0, β-endorphin (1-17) (net charge = +2) was well separated from β-endorphin (1-9) (net charge = +2) using 50% AcCN but not 25% AcCN, whereas in the same mobile phase the separation of β-endorphin (1-9) (net charge = +2) from β-endorphin (1-16) (net charge = +2) was better in 10% AcCN than in 25% AcCN (1). The separation of crude peptide A mixture using different AcCN concentrations is shown in Figure 15.1. The highest resolution value was obtained at 35% AcCN. However, minor imprities eluted as shoulders on the main peaks at this AcCN concentration, and therefore this condition was not examine further. Lower levels of acetonitrile (10% and 25%) gave better separation of these minor impurities than the higher level (35%) and 10% AcCN was selected to separate the peptides on the analytical SCX column (Figure 15.2).

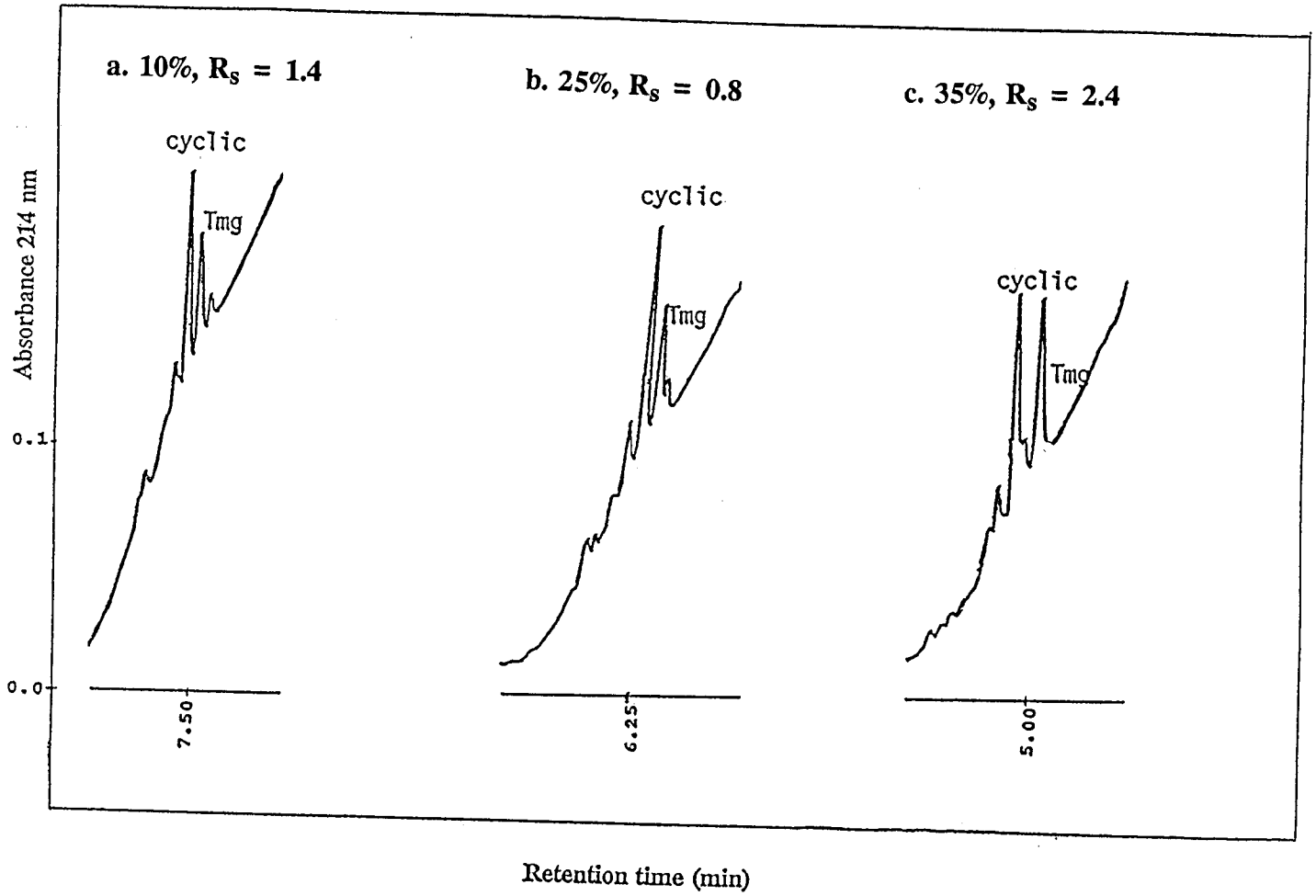


Figure 15.1 Effects of organic modifiers on IEC. Sample: crude peptide A. Column: SCX guard cartridge. Mobile phase: solvent A = 5 mM KH_2PO_4+ x% AcCN pH 3.0, a) x = 10 b) x = 25 and c) x = 35, solvent B = solvent A + 800 mM KCl. Flow rate: 1.5 mL/min. Gradient: 10% to 70% B over 12 min.

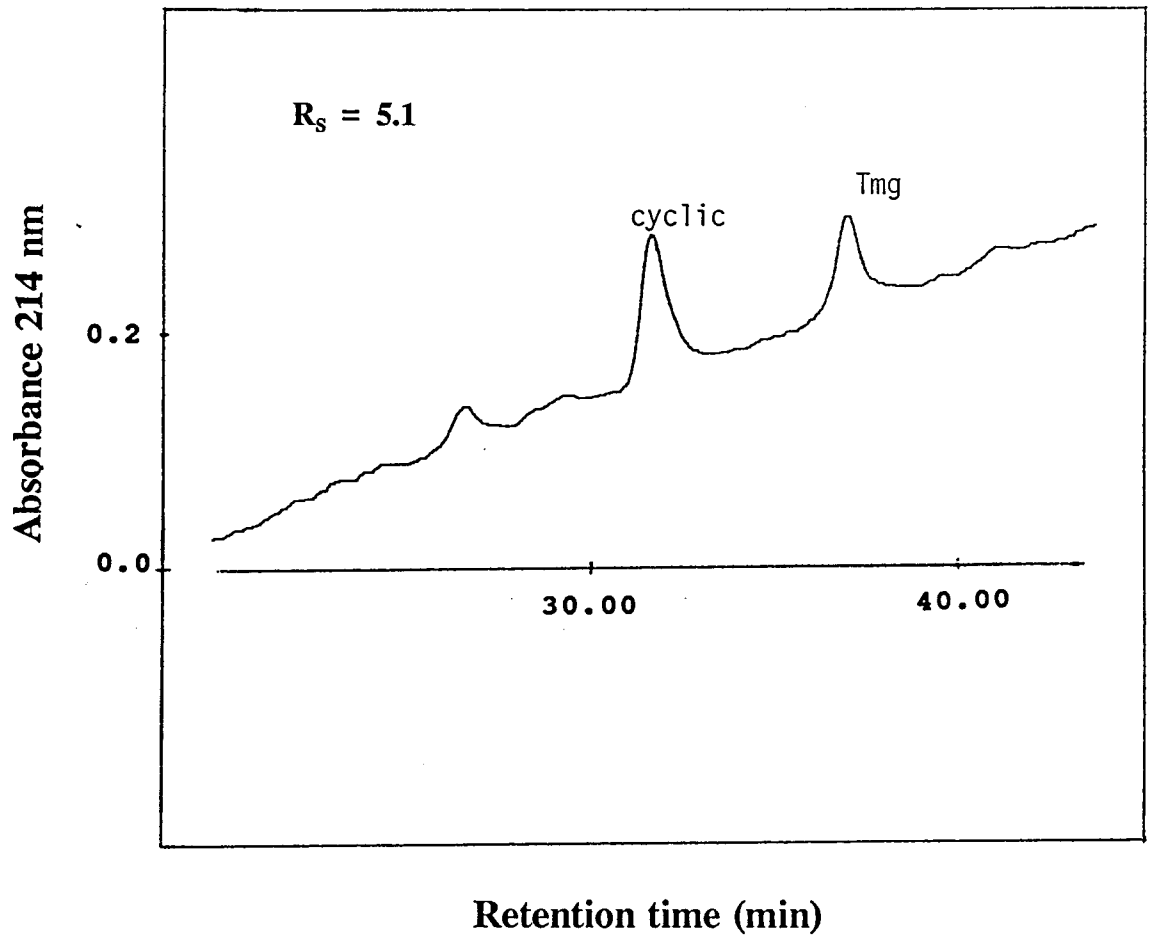


Figure 15.2 The separation of crude peptide A mixture on the analytical SCX column. Mobile phase: solvent A = 5 mM KH_2PO_4 + 10% AcCN pH 3.0, solvent B = solvent A + 800 mM KCl. Flow rate 1.5 mL/min. Gradient: 20% to 70% B over 100 min.

Loading study on SCX column

The optimized conditions from the method development, namely phosphate buffer, pH 3.0, containing 10% AcCN with an increasing salt gradient, were used in a loading study to determine the capacity of a preparative column. Results of the loading capacity on the SCX column in Figure 16 indicate that a 1 mg loading gave good resolution; scaling the loading up to 5 mg caused some decrease in resolution, but still gave a reasonable separation. The cyclic peptide and Tmg byproduct could not be resolved, however, when the column was overloaded with 10 mg of crude peptides. Therefore, the loading capacity of a half inch diameter preparative SCX column is approximately 50 mg.

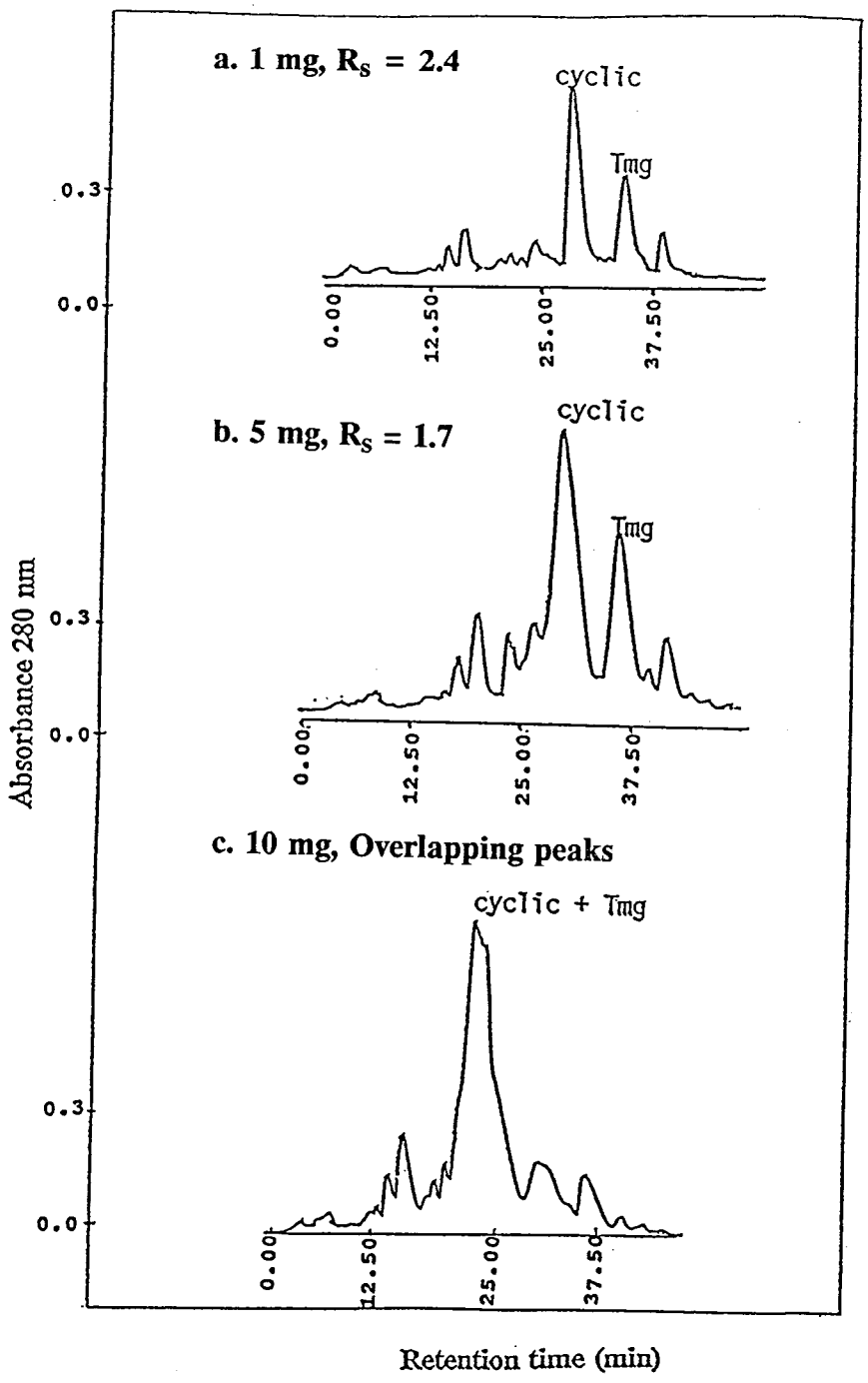


Figure 16. Loading studies on SCX column. Sample: crude peptide A a) 1 mg, b) 5 mg and c) 10 mg. Column: SCX column. Mobile phase: solvent A = 5 mM KH_2PO_4+10% AcCN pH 3.0, solvent B = solvent A + 800 mM KCl. Flow rate: 1.5 mL/min. Gradient: 20% to 60% B over 80 min.

Table 8.1. Retention times of purified peptides under the RPC conditions used for the separation of crude peptide A.

Condition	t_{cyclic} (min)[1]	t_{Tmg} (min)[2]	t_{linear} (min)[3]
Figure 10a	17.9	16.7	14.8
10b	25.3	25.3	24.4
10c	13.2	9.8	9.4
10d	14.7	11.3	11.3
11a	14.7	11.3	11.3
11b	-[4]	-[4]	-[4]
12.1a	26.4	22.2	19.9
12.1b	17.9	16.0	16.5
12.2a	22.4	15.7	13.6
12.2b	14.7	11.3	11.3
13.1a	14.7	11.3	11.3
13.1b	19.6	13.8	10.7
13.2	22.0	16.2	12.9

[1,2 and 3]Retention time of purified cyclic peptide cyclo[D-Asp^2,Orn^5]Dyn A(1-13)NH_2, Tmg byproduct and linear byproduct, respectively.
[4]Very broad peak

Table 8.2 Retention times of purified peptides under the IEC conditions used for the separation of crude peptide A.

Condition	t_{cyclic} (min)[1]	t_{tmg} (min)[2]	t_{linear} (min)[3]
Figure 14a	6.0	6.5	5.8
14b	3.6	4.0	3.9
15.1a	7.0	7.7	7.2
15.1b	6.0	6.5	5.8
15.1c	4.6	5.4	7.1
15.2	31.3	36.6	38.1

[1,2 and 3]Retention time of purified cyclic peptide cyclo[D-Asp^2,Orn^5]Dyn A(1-13)NH_2, Tmg byproduct and linear byproduct, respectively.

Table 9.1. Resolution values (R_s) of the cyclic peptide and the Tmg byproduct for the separation of crude peptide A mixture under various RPC conditions.

Condition	t_{cyclic} (min)[1]	t_{Tmg} (min)[2]	Δ t(min)[3]	R_S[4]	R_s[5]
Figure 10a	18.2	16.2	2.0	4.7	1.8
10b	-[6]	-[6]	-[6]	-[6]	-[6]
10c	12.8	9.9	2.9	8.6	3.2
10d	14.8	11.3	3.5	10.3	3.5
11a	14.8	11.3	3.5	10.3	3.5
11b	-[6]	-[6]	-[6]	-[6]	-[6]
12.1a	26.2	22.3	3.9	5.7	3.3
12.1b	18.2	16.2	2.0	4.7	1.8
12.2a	22.2	14.6	7.6	6.7	5.1
12.2b	14.8	11.3	3.5	10.3	3.5
13.1a	14.8	11.3	3.5	10.3	3.5
13.1b	19.3	14.1	5.2	7.7	3.7
13.2	22.1	16.4	5.7	11.2	5.7
13.3a	21.1	15.7	5.4	7.1	8.3
13.3b	30.2	22.6	7.6	3.7	5.4

[1 and 2]Retention of cyclic peptide and Tmg byproduct, respectively.

[3]$t_{cyclic} - t_{Tmg}$.

[4]Calculated by using peak width at half height (equation (b)).

[5]Calculated by using baseline peak width (equation (a)).

[6]Overlapping peaks.

Table 9.2 Resolution values (R_s) of the cyclic peptide and the Tmg byproduct for the separation of crude peptide A mixture under various IEC conditions.

Condition	t_{cyclic} (min)[1]	t_{Tmg} (min)[2]	Δ t (min)[3]	R_S[4]	R_S[5]
Figure 14a	6.0	6.5	0.5	0.8	0.9
14b	-[6]	-[6]	-[6]	-[6]	-[6]
15.1a	7.4	8.0	0.6	1.4	1.2
15.1b	6.0	6.5	0.5	0.8	0.9
15.1c	4.7	5.5	0.8	2.4	0.8
15.2	31.1	36.3	5.2	5.1	2.9
16a	28.3	34.3	6.1	2.4	5.3
16b	29.1	35.8	6.7	1.7	2.2
16c	-[6]	-[6]	-[6]	-[6]	-[6]

[1 and 2]Retention time of cyclic peptide and Tmg byproduct, respectively.
[3]$t_{Tmg} - t_{cyclic}$
[4]Calculated by using peak width at half height (equation (b)).
[5]Calculated by using baseline peak width (equation (a)).
[6]Overlapping peaks.

CONCLUSIONS

The separation of the cyclic analogue of dynorphin A from the Tmg side product can be achieved by either reverse phase or ion exchange chromatography. In RPC, the Tmg byproduct was eluted prior to the desired cyclic peptide under all of the chromatographic conditions examined except when HFBA was used as the ion-pairing agent. Four possible solvent systems of RPC gave the best results, 0.1% TFA in aqueous MeOH, 0.1% H_3PO_4 in aqueous AcCN, TEAP (I) in aqueous AcCN and TEAP(II) in 60% AcCN (e.g. mobile phase 2, 4, 5 and 6, respectively). Using phosphoric acid as the ion-pairing agent (mobile phase 4) or TEAP buffer (mobile phases 5 and 6) requires a desalting step, whereas pure fractions can be obtained directly from 0.1% TFA in MeOH (mobile phase 2). The loading capacity on the Zorbax column using mobile phase 6 (0.09 N TEAP in 60% AcCN) is 1-3 mg, which is equivalent to 20-60 mg on the one inch preparative column available in our laboratory.

In IEC, a phosphate buffer, pH 3.0, containing 10% acetonitrile with an increasing gradient of KCl provided optimum conditions for the separation of crude peptide A. The amount of salt required to elute the cyclic peptide and Tmg byproduct on SCX column was 350-400 mM and the cyclic peptide was eluted before the Tmg byproduct. During IEC, the loading capacity of the analytical SCX column was 5 mg, which is equivalent to 50 mg on a half-inch preparative column.

An advantage of RPC over IEC for preparative purification of peptides is that a subsequent desalting step is not required if TFA is used as the ion pairing agent since TFA is volatile. If a phosphate-containing buffer such as TEAP is used for RPC, however, an additional desalting step is needed. A second advantage is that the mobile phases used in RPC do not contain a high salt concentration, and therefore will not damage standard steel HPLC systems.

The advantage of IEC over RPC is that the cyclic peptide is eluted prior to the Tmg byproduct resulting in less contamination from IEC purification. However, the high concentration of salt (400 mM KCl) requires a desalting step in the final purification and the high percentage of salt can damage standard HPLC systems. Thus, a non-steel system is preferred when using ion exchange chromatography.

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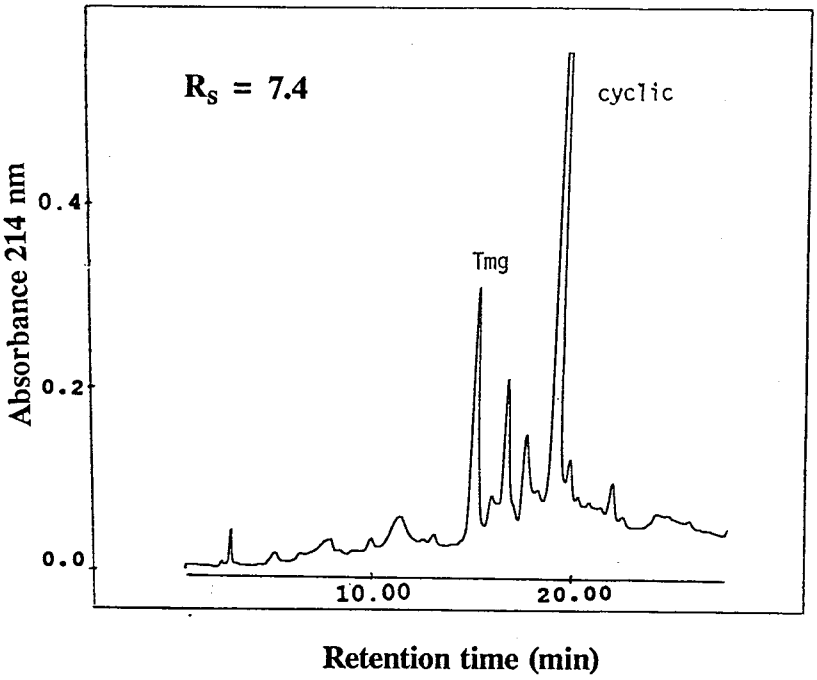
APPENDICES

Appendix A. Resolution values (R_S) of the cyclic peptide and the Tmg byproduct for the separation of crude peptide A on different RPC columns under the same conditions as in Figure 13.2 (TEAP (II) buffer, pH 2.5 in 60% AcCN).

Column	t_{cyclic} (min)[1]	t_{Tmg} (min)[2]	Δt (min)[3]	R_S[4]	R_S[5]
Vydac	19.3	14.1	5.2	7.7	3.7
Zorbax[6]	22.1	16.4	5.7	11.2	5.7
Dynamax	24.0	19.2	4.8	11.3	4.8

[1 and 2]Retention time of the cyclic peptide and the Tmg byproduct, respectively.
[3]$t_{cyclic} - t_{Tmg}$
[4]Calculated by using peak width at half height (equation (b)).
[5]Calculated by using baseline peak width (equation (a)).
[6]This column was used for Figure 13.2.

Appendix B. Chromatogram of the separation of crude peptide B under the same conditions as in Figure 13.2 (Zorbax column using TEAP (II) buffer, pH 2.5 in 60% AcCN).

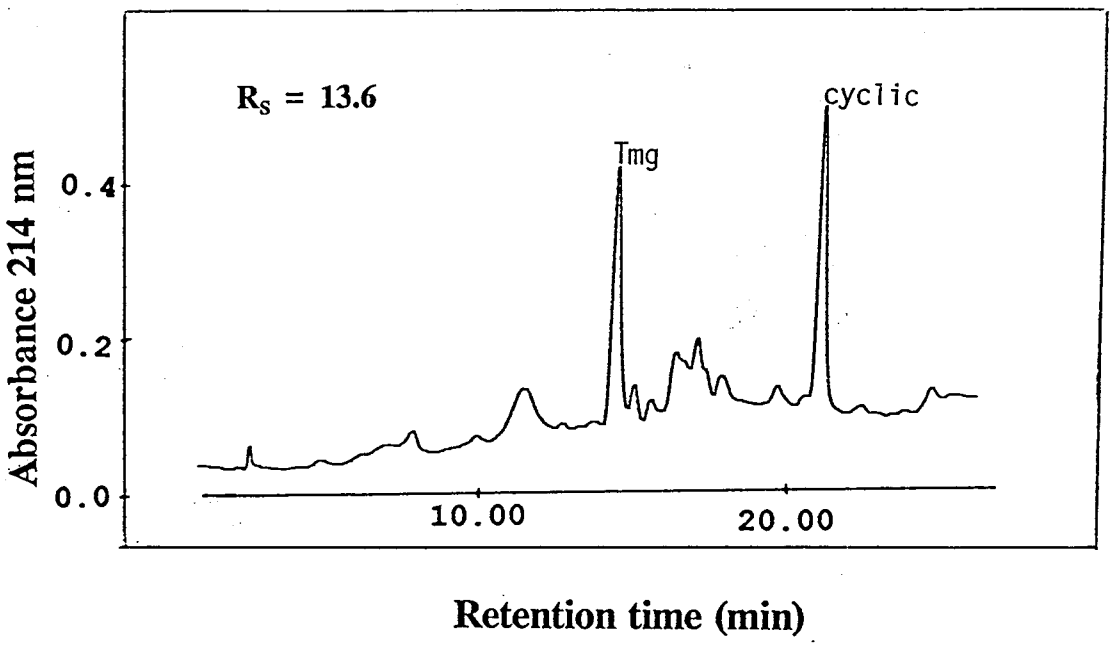


Appendix C. Resolution values (R_S) of the cyclic peptide and the Tmg byproduct for the separation of crude peptide B on different RPC columns under the same conditions as in Figure 13.2 (TEAP (II) buffer, pH 2.5 in 60% AcCN).

Column	t_{cyclic} (min)[1]	t_{Tmg} (min)[2]	Δt (min)[3]	R_S[4]	R_S[5]
Vydac	16.4	12.7	3.7	5.5	2.2
Zorbax[6]	19.8	15.4	4.4	7.4	3.1
Dynamax	21.9	18.2	3.7	10.9	4.1

[1 and 2]Retention time of the cyclic peptide and the Tmg byproduct, respectively.
[3]$t_{cyclic} - t_{Tmg}$
[4]Calculated by using peak width at half height (equation (b)).
[5]Calculated by using baseline peak width (equation (a)).
[6]This column was used for Figure 13.2.

Appendix D. Chromatogram of the separation of crude peptide C under the same conditions as in Figure 13.2 (Zorbax column using TEAP (II) buffer, pH 2.5 in 60% AcCN).



Appendix E. Resolution values (R_S) of the cyclic peptide and the Tmg byproduct for the separation of crude peptide C on different RPC columns under the same conditions as in Figure 13.2 (TEAP (II) buffer, pH 2.5 in 60% AcCN).

Column	t_{cyclic} (min)[1]	t_{Tmg} (min)[2]	Δt (min)[3]	R_S[4]	R_S[5]
Vydac	15.3	12.1	3.2	7.6	2.3
Zorbax[6]	21.4	14.5	6.9	13.6	6.9
Dynamax	23.7	17.8	5.9	17.4	6.6

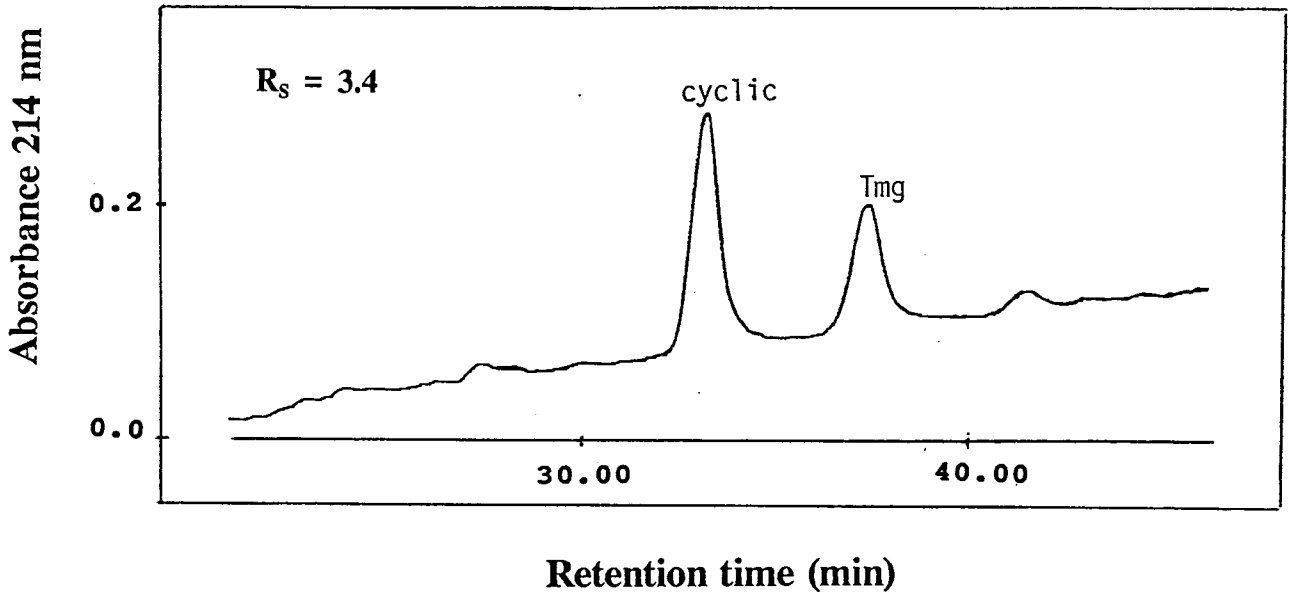
[1 and 2]Retention time of the cyclic peptide and the Tmg byproduct, respectively.
[3]$t_{cyclic} - t_{Tmg}$
[4]Calculated by using peak width at half height (equation (b)).
[5]Calculated by using baseline peak width (equation (a)).
[6]This column was used in Figure 13.2.

Appendix F. Resolution values (R_S) of the cyclic peptide and the Tmg byproduct for the separation of different crude peptides on SCX column under the same conditions as in Figure 15.2 (phosphate buffer, pH 3.0 containing 10% AcCN with KCl gradient).

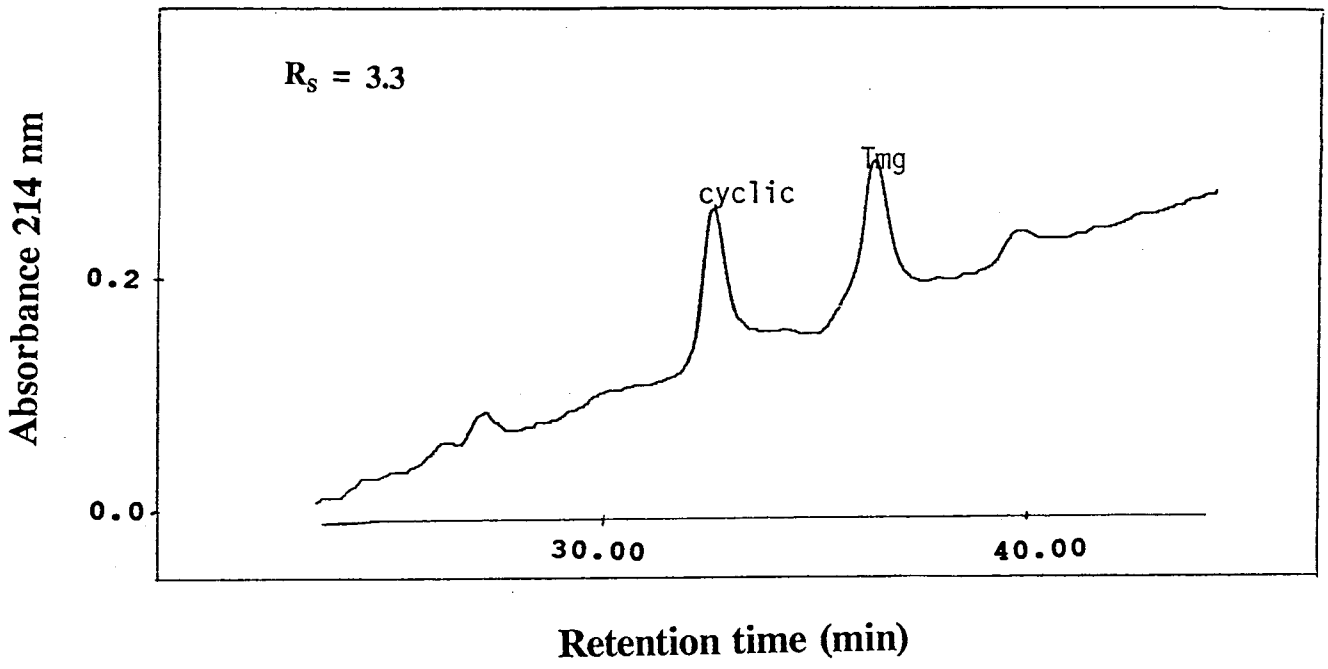
Crude peptide	t_{cyclic} (min)[1]	t_{Tmg} (min)[2]	Δt (min)[3]	R_S[4]	R_S[5]
A	31.1	36.3	5.2	5.1	2.9
B	32.8	37.1	4.3	3.4	2.0
C	31.8	36.0	4.2	3.3	1.6

[1 and 2]Retention time of the cyclic peptide and the Tmg byproduct, respectively.
[3]$t_{Tmg} - t_{cyclic}$
[4]Calculated by using peak width at half height (equation (b)).
[5]Calculated by using baseline peak width (equation (a)).

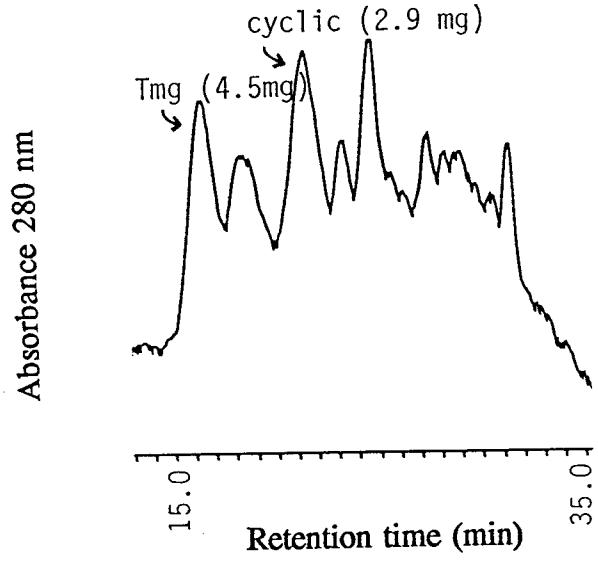
Appendix G. Chromatogram of the separation of crude peptide B on SCX column under the same conditions as in Figure 15.2 (phosphate buffer, pH 3.0 containing 10% AcCN with KCl gradient).



Appendix H. Chromatogram of the separation of crude peptide C on SCX column under the same conditions as in Figure 15.2 (phosphate buffer, pH 3.0 in 10% AcCN with KCl gradient).



Appendix I. Chromatogram of the separation of crude peptide A (45.9 mg) on the preparative Zorbax Protein Plus (21 x 250 mm) using 0.1% TFA in aqueous MeOH. Flow rate: 20 mL/min. Gradient: 20% to 50% over 30 min.



Appendix J. Chromatogram of the separation of crude peptide A (17.9 mg) on the preparative Zorbax Protein Plus (21 x 250 mm) using TEAP II buffer, pH 2.5 containing 60% AcCN. Flow rate: 20 mL/min. Gradient: 0% to 60% over 60 min. TEAP desalting was done on Zorbax column using solvent system 1 (Table 5). The salt was washed with 100% solvent A for 10 min and then the peptide was eluted with 18% solvent B. Flow rate: 1.5 mL/min.